VOLUME 39 SEPTEMBER 1961 NUMBER 9

Canadian Journal of Biochemistry and Physiology

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Published by THE NATIONAL RESEARCH COUNCIL
OTTAWA CANADA

Canadian Journal of Biochemistry and Physiology

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CORRECTIONS

Volume 38, 1960

Page 376. Under "Results", in fifth line from the bottom, "2.5×10^{-8} g/liter" should be changed to read "2.5×10^{-8} g/ml".

Page 380. In Fig. 4 caption, "2.5×10^{-8} g/liter" and "7.5×10^{-7} g/liter" should be changed to read "2.5×10^{-8} g/ml" and "7.5×10^{-7} g/ml" respectively.

Volume 39, 1961

Page 345. In Table I, the heading for the fourth column (from the left) should be changed to read "1/C.V.".

Page 1129. In Table III, the heading "Biological half-life (hours)" should be changed to read "Biological half-life (days)".

Page 1310. In tenth line from the bottom, "to precipitate the calcium (1)" should be changed to read "to precipitate the calcium (1, 2, 3)".

Canadian Journal of Biochemistry and Physiology

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOLUME 39 SEPTEMBER 1961 NUMBER 9

SOME PROPERTIES OF THE SOLUBLE ESTERASES OF HUMAN LIVER[1]

D. J. ECOBICHON AND W. KALOW

Abstract

Zone electrophoresis on starch gel in conjunction with various histochemical staining methods was applied to the study of the water-soluble esterases of liver. The results indicated that in regard to electrophoretic migration and enzymatic properties, none of the human liver esterases was identical with any of the human serum esterases.

Starch gel electrophoresis has been used to study the esterases of mouse and rat liver (1), of human serum (2), of maize (3), of unicellular organisms (4), and of the silk moth (5). The present paper deals with the application of this method to the water-soluble enzymes of human liver.

Methods

The starch was prepared according to Smithies (6, 7), usually with the modification that a 0.006 *M* phosphate buffer (1.85 g $Na_2HPO_4.12H_2O$ and 0.2 g KH_2PO_4 per liter, pH 7.2) was employed in the preparation of the gel and as the bridge buffer. Electrophoresis was carried out for a period of 16 hours with a current of 20 to 25 milliamperes (100–110 volts) passing through the gel.

Samples of human liver were obtained at autopsy from unselected cases in the pathological laboratory of a general hospital. The tissues were homogenized in a Potter–Elvehjem homogenizer with tolerances of 0.025 mm in the presence of sufficient distilled water to produce final concentrations of 4 g in 40 ml of homogenate. These were frozen slowly, thawed, and centrifuged at 11,000×*g* for 1 hour in a refrigerated centrifuge at 0° C. The sediment was discarded and the supernatant, in a viscose dialysis bag, was placed in a tray and covered with polyethylene glycol (Carbowax). This removed sufficient

[1]Manuscript received March 2, 1961.
Contribution from the Department of Pharmacology, University of Toronto, Toronto, Ontario.

water from the sample to produce a final volume of 5 ml. Human serum was used for comparison with the liver preparations. The staining and migrating properties of the serum esterases remained unchanged if subjected to the same treatment as the liver tissue. All samples were kept in a frozen state until just before use.

After electrophoresis, the gel was cut into two equal slices as in Smithies' procedure (7), and was stained to localize proteins or esterases. Amido black B was used to locate the proteins. The position of the esterases was demonstrated by employing the substrates listed in Table I; the first four of these gave hydrolysis products which coupled with naphthanil diazo blue B for the development of color (8). The substrates were used in concentrations ranging from 5×10^{-4} *M* to 1×10^{-3} *M*. In order to characterize further the esterases, the gel was exposed to the inhibitor solution (inhibitors and concentrations used are in Table I) for 20 minutes before staining.

The serum esterases on the starch gel were identified by eluting the enzymes from the starch and measuring their activity in solution in a recording spectrophotometer. The method of elution and the criteria for the identification of pseudocholinesterase have been described (2). Aromatic esterase was eluted in the same manner and identified by the properties described by Marton and Kalow (9, 10).

Results

Figure 1 shows the localization of the two esterases of human serum (10, 11), i.e. aromatic esterase (zone S-1) and pseudocholinesterase (zone S-2). The identity of these two enzymes was firmly established after they had been eluted from the gel. The distance of migration of these two zones are different from any of the liver enzymes which are found in three distinct zones. Zone L-3 of liver enzymes consists of three bands which have migrated toward the cathode rather than the anode. Zones L-1 and L-2 of liver are also composed of bands which are only sometimes visible, and then immediately after the beginning of the staining reaction, but they do not show in Fig. 1. Zone L-3 of liver does not appear if a buffer at pH 8.3 is used.

On occasion, an esterase corresponding to that of zone S-2 was found in the liver preparation. The staining reactions were faint enough to suggest that this was due to serum trapped in the liver tissue. Furthermore, this esterase did not show when the sample was taken from the drained upper portion of a bottled specimen of liver. This observation was taken as a confirmation that the method of treatment did not alter the enzyme.

In the investigation of 76 individual livers, a large person-to-person variation has been found in the rate of migration of liver zone L-2. The ratios of fronts of zone L-2 to those of zone S-2 ranged from 0.53 to 1.24 in a continuous variation. The cause of this variation is at present not known and is under further investigation.

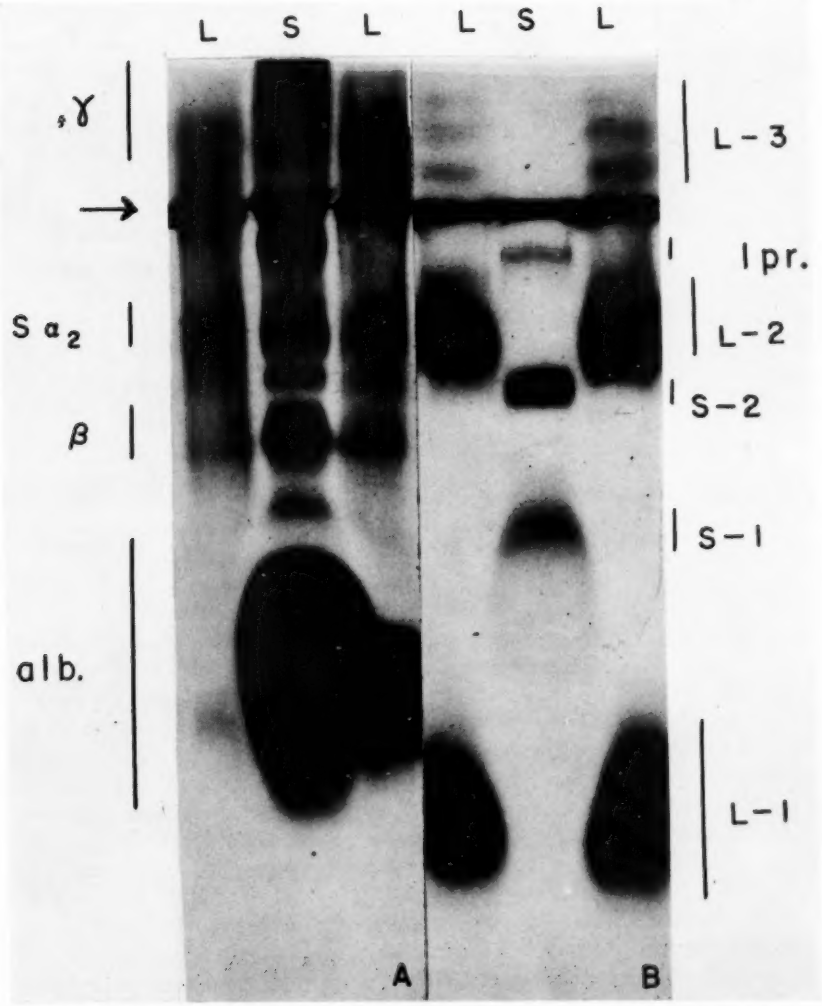

FIG. 1. Proteins and esterases of human liver homogenate and serum in starch gel after electrophoresis. Part A: The protein pattern as developed with amido black B. Part B: The enzyme pattern as developed with beta-naphthyl acetate and naphthanil diazo blue B. Abbreviations L and S indicate liver and serum samples; γ, $S\alpha_2$, and β indicate the respective globulins; alb. = albumin, lpr. = lipoprotein of serum. Zones L-1, L-2, and L-3 indicate liver esterases; zone S-1 is A-type esterase; zone S-2 is cholinesterase of human serum. The arrow points to the slot cut out in the gel for the sample, i.e. the origin. Anodes is towards the bottom of the picture.

Ecobichon and Kalow—Can. J. Biochem. Physiol.

The results obtained by using different substrates and inhibitors are presented in Table I.

TABLE I

The activity of the esterase zones of liver and serum in starch gels

Substrates (and inhibitors)	Liver zones			Serum zones	
	L-1	L-2	L-3	S-1	S-2
alpha-Naphthyl acetate	+	+	+	+	+
beta-Naphthyl acetate (1)	+	+	+	+	+
beta-Carbonaphthoxycholine iodide (8)	−	+	−	−	+
Naphthol AS acetate (1)	−	+	+	−	+
Indoxyl acetate (12)	+	+	+	−	+
5-Bromoindoxyl acetate (13)	+	+	+	−	+
Acetylthiocholine (14)	−	−	−	−	+
beta-Naphthyl acetate (omission of calcium)	+	+	+	−	+
beta-Naphthyl acetate with calcium and one of the following:					
Eserine (1×10^{-4} *M*)	−	+	−	+	−
R02-0683 (4×10^{-6} *M*) (15)	−	+	−	+	−
BW 284c51 (1.75×10^{-5} *M*) (16)	−	+	−	+	−
BW 62c47 (1.75×10^{-5} *M*) (16)	−	+	−	+	−
TEPP (3.6×10^{-5} *M*) (17)	−	+	−	+	−

NOTE: + indicates the presence, − the absence, of a staining reaction as a result of esterase activity. The location of the zones is shown in Fig. 1. Inhibitors used were as follows:
R02-0683 = dimethyl carbamate of 2-hydroxy-5-phenylbenzyl trimethylammonium bromide,
BW 284c51 = 1,5-bis(4-allyl dimethylammonium phenyl)pentan-3-one diodiide,
BW 62c47 = 1,5-bis(4-trimethylammonium phenyl)pentan-3-one diiodide,
TEPP = tetraethylpyrophosphate.
Literature references are indicated in parentheses.

Discussion

Considering the data of Table I, the serum zones behave as one might expect: the pseudocholinesterase (zone S-2) hydrolyzes all investigated esters including the two choline esters and it is inhibited by the five blocking agents, which are all known to be anticholinesterases. The fact that two of these five inhibitors would preferentially inhibit acetylcholinesterase (16), and the other three, pseudocholinesterase (15, 17), does not seem to matter under the chosen experimental conditions. Serum zone S-1 contains aromatic esterase. It hydrolyzes beta-naphthyl acetate but does not attack choline esters. No specific inhibitor of this enzyme is known but it can function only in the presence of calcium (11).

None of the liver zones shows the same properties as either of the two serum esterases. One may note specifically that zones L-1 and L-3 are blocked by the anticholinesterases but they do not hydrolyze choline esters. On the other hand, beta-carbonaphthoxycholine iodide is supposed to be a specific substrate for pseudocholinesterase (8, 11), but it is hydrolyzed by an enzyme which is resistant to the action of all anticholinesterases. One may wonder whether any of the liver enzymes could be aliesterase (B-type esterase), an enzyme which occurs in many animals but which is absent from human serum

(9, 18). Aliesterase is characterized by resistance to eserine, though being susceptible to inhibition by TEPP. However, no enzyme with such features has been observed in the present series.

Pseudocholinesterase has been presumed to be derived from the liver as cellular damage in the liver causes a lowering of serum pseudocholinesterase levels. There is evidence of some parallelism between albumin and pseudocholinesterase levels in serum. In cases of liver damage, albumin which is formed in liver is lowered, as is the pseudocholinesterase level (19). In view of this postulated relationship, it appears remarkable that liver does not contain a pseudocholinesterase in a readily soluble form.

The methods used are not suitable for a full characterization of these enzymes. The present results are of a qualitative nature but indicate the necessity for a complete restudy of purified liver esterases by quantitative tests.

Acknowledgments

This work was supported by a grant (MA 712) from the National Research Council of Canada.

We are grateful to Hoffmann-LaRoche, Montreal, for gifts of R02-0683, and to the Wellcome Research Laboratories, Tuckahoe, New York, for gifts of BW 62c47 and BW 284c51.

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HYDROLYSIS OF SOME DEOXYRIBONUCLEOSIDES BY WHEAT LEAF JUICE[1]

D. W. A. ROBERTS

Abstract

Enzymes present in wheat leaf juice convert deoxyadenosine into adenine and deoxyribose, deoxycytidine into deoxyuridine, and 5-methyldeoxycytidine into thymidine.

Introduction

Wheat leaves contain at least two enzymes that hydrolyze ribonucleosides. Adenosine is converted into adenine and ribose, and cytidine, into uridine and ammonia (1). Uridine, guanosine, and inosine are not affected at pH 5.5 by enzymes present in juice from wheat leaves. Tests have now been made with the deoxy analogues of these compounds. Since an enzyme from *Escherichia coli* is reported to deaminate 5-methyldeoxycytidine (2, 3) this compound was also tested.

Materials and Methods

The wheat leaf juice and the enzyme digests were prepared as described earlier (1). Descending paper chromatography with six solvent systems and Whatman No. 1 filter paper was used to identify the reaction products. Authentic samples were chromatographed on the same sheets as the unknowns. The following solvent systems were used: (*a*) *n*-butanol : acetic acid : water 5:4:1; (*b*) *n*-propanol : ammonium hydroxide, specific gravity 0.90 : water, 3:1:1 (4); (*c*) *tert*-butyl alcohol : water : formic acid, specific gravity 1.2, 16:4:1 (4); (*d*) isobutyric acid : water : acetic acid, 100:50:1 (5); (*e*) isobutyric acid : water : ammonium hydroxide, specific gravity 0.90, 60:33:1 (6); and (*f*) *n*-propanol : tetrahydrofurfuryl alcohol : water : 0.75 *M* citrate pH 5.7, 40:20:18:2 (7). R_F data for the relevant compounds in these solvents have been published (8). The deoxyribose and deoxyribosides were detected with the Dische reagent (9). This spray did not give satisfactory results on chromatograms irrigated with solvent (*f*) on account of excessive background color development. The purine and pyrimidine derivatives were detected with ultraviolet light.

In those cases in which the enzymes produced a new compound from the substrate supplied, the area on the filter paper chromatogram that contained the new substance was cut out and eluted with 0.01 *N* hydrochloric acid. The ultraviolet-absorption spectra in 0.01 *N* HCl of these eluates were determined using the eluate from a small adjacent area of chromatogram as a blank.

[1]Manuscript received March 1, 1961.
Contribution from the Plant Pathology Section, Canada Agriculture Research Station, Lethbridge, Alberta, Canada.

Spectra of the reaction products were compared with those of known compounds that had been chromatographed on, and eluted from, the same sheet of filter paper.

Results

Only unchanged deoxyguanosine, deoxyinosine, deoxyuridine, and thymidine could be detected after these compounds were incubated with dialyzed wheat leaf juice for periods up to 2 days at pH 5.5. When deoxyadenosine was added to the dialyzed juice, irrigation of aliquots with solvent (*b*) revealed the presence of free adenine and with solvents (*a*), (*d*), and (*e*), the presence of deoxyribose. In the case of deoxycytidine, irrigation with solvents (*c*), (*e*), and (*f*) showed that deoxyuridine was present in aliquots, while deoxyribose was not detected on chromatograms irrigated with solvent (*e*) nor cytosine on those irrigated with solvents (*b*) and (*f*). With samples from 5-methyldeoxycytidine digests, irrigation with solvents (*b*), (*c*), (*d*), and (*f*) revealed the presence of thymidine. The Dische reagent failed to detect any free deoxyribose even in preparations irrigated with solvent (*e*), which should have separated the deoxyribose from the other compounds present.

The ultraviolet-absorption spectra over the range from 220 mμ to 320 mμ for the compounds identified by paper chromatography as adenine, deoxyuridine, and thymidine confirmed these identifications.

Discussion

The data show that enzymes in wheat leaf juice convert deoxyadenosine into adenine and deoxyribose, deoxycytidine into deoxyuridine, and 5-methyldeoxycytidine into thymidine. Earlier (1) it was shown that adenosine is converted into adenine and ribose and that cytidine is converted into uridine by similar wheat leaf juice preparations. Until the enzymes that catalyze these reactions are purified it is reasonable to assume that only two enzymes, namely adenosine ribosidase and cytidine deaminase, are responsible for these conversions.

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LOCALIZATION AND CHARACTERIZATION OF CHOLINESTERASE IN SUBCELLULAR FRACTIONS OF RAT BRAIN AND BEEF PITUITARY[1]

SURENDRA S. PARMAR, MORLEY C. SUTTER, AND MARK NICKERSON

Abstract

Fresh rat brains and fresh anterior and posterior pituitary glands of beef were separated by differential centrifugation into subcellular fractions, characterized on the basis of sedimentation and succinic dehydrogenase activity. Cholinesterase activity was measured by both manometric and colorimetric methods, the results of which were comparable. Cholinesterase activity of rat brain was found mainly in the microsome and supernatant fractions. It was quite uniformly distributed in all subcellular fractions of both anterior and posterior pituitary. Comparisons of the relative rates of hydrolysis of acetylthiocholine and butyrylthiocholine, and of inhibition by eserine, indicated that brain contains a much higher percentage of acetylcholinesterase than do both lobes of the pituitary, which contain relatively low concentrations of the specific enzyme. Total cholinesterase activity and its sensitivity to inhibition by eserine in the posterior pituitary were found to be midway between those of the anterior lobe and of the brain, from which the posterior pituitary was derived during embryological development.

Introduction

A number of esterases are known to be capable of hydrolyzing acetylcholine (1, 2, 3, 4), but data on their classification and intracellular localization are incomplete. Alles and Hawes (5) reported differences between various cholinesterases on the basis of their substrate specificities. Mendel and Rudney (6, 7) extended this work and introduced the term pseudocholinesterase to describe the enzyme found in serum, which exhibits maximum activity at high concentrations of acetylcholine and hydrolyzes benzoylcholine and butyrylcholine, but not acetyl-β-methylcholine. Augustinsson and Nachmansohn (8) studied the distinction between acetylcholinesterase and other enzymes capable of splitting choline esters. They designated the enzyme present in brain and erythrocytes as "true" or "specific" cholinesterase or acetylcholinesterase. This enzyme displays maximum activity at low concentrations of acetylcholine and hydrolyzes acetyl-β-methylcholine, but not benzoylcholine or butyrylcholine.

The localization and characterization of cholinesterases at a subcellular level have been studied histochemically, using thioesters of choline, and by differential centrifugation of tissue homogenates. Koelle, using histochemical methods, found that most of the true cholinesterase in brain is associated with the cell membranes (9) and synaptic areas of neurones (10). Several other workers (11, 12, 13, 14) have attempted to characterize the cholinesterases

[1]Manuscript received February 22, 1961.
Contribution from the Department of Pharmacology and Therapeutics, University of Manitoba Faculty of Medicine, Winnipeg, Manitoba. Supported by grants-in-aid from the National Research Council of Canada and the Life Insurance Medical Research Fund.

found in various subcellular fractions of brain obtained by differential centrifugation. The present study was undertaken to determine the intracellular distribution and to characterize the cholinesterases in brain and pituitary, and to compare enzyme in the posterior pituitary with that in the functionally related anterior pituitary and the embryologically related brain.

Materials and Methods

Homogenization

Adult rats weighing 200 to 250 g were killed by decapitation, the brains removed, placed in a container surrounded by ice, and processed in the cold room. Beef pituitaries were obtained from a slaughter house, transported in containers surrounded by ice, and anterior and posterior lobes separated in a cold room. Tissues were homogenized in ice-cold 0.25 *M* sucrose using a motor-driven Teflon–Pyrex homogenizer with the chamber clearance of 0.004 to 0.006 inch. The final concentration of the homogenate was 10% w/v. All preparative procedures were carried out in a cold room at 4° C, and the centrifugal fractionation done in a Servall refrigerated centrifuge.

Fractionation Procedures

The rat brain homogenate was fractionated by differential centrifugation using a modification of the techniques described by Aldridge (15) and Brody and Bain (16). Nuclei and cell debris were obtained by centrifugation at 600×*g* for 10 minutes, mitochondria at 10,000×*g* for 15 minutes, and microsomes at 30,000×*g* for 2 hours. Each of the fractions except microsomes was washed and recentrifuged twice and then suspended in a known volume of 0.25 *M* sucrose. The wash fluid from each fraction was added to the supernatant prior to the next stage of centrifugation, and the supernatant from the preparation of the microsome fraction was designated as the supernatant fraction.

The anterior pituitary gland homogenates were fractionated by the method of LaBella and Brown (17). Nuclei and cell debris were obtained by centrifugation at 700×*g* for 15 minutes, secretory granules at 7000×*g* for 10 minutes, mitochondria at 20,000×*g* for 10 minutes, and microsomes at 30,000×*g* for 3 hours. The fractions were washed and the wash fluids added to the next step until the final supernatant fraction was obtained as in the case of brain.

In the preparation of subcellular fractions from posterior pituitary gland, heparin solution at a final concentration of 200 μg/ml was added to the supernatant obtained after separation of nuclei and cell debris at 600×*g* for 15 minutes. This concentration of heparin did not affect enzyme activity; it improved the selectivity of the fractionation. Unlike those in anterior pituitary, mitochondria in posterior pituitary homogenates sedimented before the secretory granules. Mitochondria were obtained by centrifugation at 14,000×*g* for 15 minutes, secretory granules at 20,000×*g* for 20 minutes, and microsomes at 30,000×*g* for 3 hours. The fractions were washed and the supernatant fraction obtained as in the case of brain and anterior pituitary gland.

Assay Procedures

(a) Succinic Dehydrogenase

The enzyme activity was determined on each fraction by the manometric method described by Schneider and Potter (18) except that adenosine monophosphate (0.0012 *M*) and adenosine triphosphate (0.0012 *M*) were added to the reaction medium. Duplicate samples were used in each determination and the values expressed as O_2 uptake in μl/100 mg of tissue/30 minutes, corrected for endogenous O_2 utilization.

(b) Cholinesterase

1. *Manometric method.*—Enzyme activity in the various fractions was determined in a Warburg apparatus at 37° C in a medium containing final concentrations of 130 mmolar NaCl, 35 mmolar $MgCl_2$, and 31 mmolar $NaHCO_3$, with a gas phase of 5% CO_2 and 95% N_2 (Augustinsson (19)). The final concentration of substrate was 20 mmolar acetylcholine, acetyl-β-methylcholine, or benzoylcholine, or 30 mmolar tributyrin. In some experiments the enzyme was incubated for 15 minutes with 1×10^{-5} or 3×10^{-5} *M* eserine sulphate prior to addition of the substrate. All determinations were done in duplicate. Enzyme activity was expressed as CO_2 evolution in μl/100 mg tissue/30 minutes or μmoles of substrate hydrolyzed, after correction for nonenzymatic hydrolysis. No CO_2 was evolved in the absence of substrate.

2. *Colorimetric method.*—Enzyme activity was determined by the method described by McOsker and Daniel (20) with acetylthiocholine or butyrylthiocholine as substrate. These thioesters were found to have the same enzyme specificity as the corresponding normal esters, which is in agreement with the observations of Koelle (21). Their hydrolysis resulted in the appearance of sulphydryl groups which were determined by the nitroprusside reaction. Each tissue sample was done in duplicate or triplicate and the results expressed as the change in optical density per 100 mg of tissue during a 10-minute incubation, or as μmoles of substrate hydrolyzed. The latter values were calculated using cysteine or reduced glutathione as a standard for the colorimetric determination of free sulphydryl groups. Measurements were made with a Beckman Model B spectrophotometer, and tissue and substrate blanks were subtracted from the readings to give the actual values due to enzymatic hydrolysis of the substrate.

Results

The subcellular distribution of succinic dehydrogenase, a mitochondrial enzyme, in the rat brain and beef anterior and posterior pituitary fractions is shown in Table I. The total succinic dehydrogenase activity recovered in these fractions was about 80 to 90% of the activity of the homogenate. The fraction designated as mitochondria contained 67 to 73% of the total recovered activity in all three tissues. The microsome fraction on the other hand contained 10 to 25% of the activity, indicating relative freedom from mitochondrial contamination.

TABLE I

Succinic dehydrogenase activity in subcellular fractions of rat brain and beef anterior and posterior pituitary gland

Tissue	Rat brain		Beef ant. pit.		Beef post. pit.	
	Oxygen uptake μl/100 mg/30 minutes					
	388		224		116	
Homogenate	368	(375)	232	(228)	121	(121)
	368		228		126	
	272		168		93	
Total recovered activity	374	(331)	176	(180)	98	(98)
	346		195		103	
	Percentage of total recovered activity					
	4		0		0	
Nuclei	1	(2)	0	(0)	0	(0)
	1		0		0	
			24		15	
Secretory granules	—		22	(23)	15	(13)
			24		10	
	60		68		67	
Mitochondria	80	(73)	67	(67)	67	(67)
	79		65		66	
	36		8		18	
Microsomes	19	(25)	11	(10)	18	(20)
	20		11		24	
	0		0		0	
Supernatant	0	(0)	0	(0)	0	(0)
	0		0		0	

NOTE: Figures in parentheses are averages.

Before the localization of cholinesterase in the various subcellular fractions was determined, it was necessary to compare the manometric and colorimetric methods. Cholinesterase activities that were determined in the various fractions of rat brain manometrically, with acetylcholine or acetylthiocholine as substrate, or colorimetrically, with acetylthiocholine as substrate, were almost identical (Table II).

Table III shows the results of studies on the distribution of cholinesterase activity in various fractions of the three tissues studied. It should be noted that the total activity was much higher in rat brain homogenate than in homogenates of either the anterior or posterior pituitary, and that the activity of the posterior was more than twice that of the anterior pituitary homogenate. All results are presented in terms of activity per unit wet weight of tissue, which was considered to be the basis of calculation most closely related to the functional state of the tissues. The nitrogen content of anterior pituitary was lower, and that of posterior pituitary higher, than that of brain. Consequently, calculations based on total nitrogen tended to decrease differences between the two lobes of the pituitary, but the order of decreasing total cholinesterase content was unchanged.

The bulk of the cholinesterase activity in rat brain was found in the microsome fraction. This was in contrast to both lobes of the pituitary, where the activity was distributed throughout all the subcellular fractions. In all three

TABLE II

Cholinesterase activity in subcellular fractions of rat brain. Comparison of manometric and colorimetric methods

	Manometric				Colorimetric	
	Acetylcholine		Acetylthiocholine		Acetylthiocholine	
	μmoles of substrate hydrolyzed/100 mg/10 minutes					
Homogenate	10.0 9.9 10.4 9.9	(10.1)	10.0 9.6 9.8 9.8	(9.8)	6.1 9.1 6.7 6.3	(7.1)
Total recovered activity	11.1 12.9 13.0 10.7	(11.9)	10.2 13.3 12.8 11.8	(12.1)	6.2 9.3 6.8 7.0	(7.3)
	Percentage of total recovered activity					
Nuclei	4.0 3.7 3.0 3.0	(3.4)	4.7 4.4 4.0 3.5	(4.1)	1.0 4.7 5.6 0.6	(3.0)
Mitochondria	6.1 12.5 14.3 15.3	(12.0)	6.3 13.3 13.0 11.8	(11.1)	15.4 6.2 5.6 14.7	(10.5)
Microsomes	50.6 44.1 43.2 45.2	(45.8)	50.9 43.8 42.9 49.5	(46.8)	49.5 51.0 49.4 51.7	(50.4)
Supernatant	39.3 39.7 39.5 36.5	(38.8)	38.1 38.5 40.1 35.2	(38.0)	34.1 38.1 39.4 33.0	(36.1)

NOTE: Figures in parentheses are averages.

tissues a relatively high percentage of the activity was present in the supernatant fraction. This is in disagreement with previous reports that the supernatant fraction of brain contains a relatively low proportion of the total activity (11, 12).

In order to determine whether variations in the fragmentation of the tissue due to chamber clearance or rotor speed of the homogenizer influenced the apparent distribution of cholinesterase, three rat brains were homogenized in chambers with clearances of 0.004 to 0.006, 0.005 to 0.007, and 0.006 to 0.009 inch. A fourth brain was homogenized using a hand-rotated pestle. The pattern of distribution of cholinesterase was identical in all fractions obtained from these four different homogenates.

Having established that most of the cholinesterase activity in rat brain was present in the microsome and supernatant fractions, these and the whole homogenate were compared with respect to their substrate specificity, using acetylcholine (hydrolyzed by a family of enzymes (1)), acetyl-β-methylcholine (relatively specific for true cholinesterase (7)), benzoylcholine (relatively specific for pseudocholinesterase (7)), and tributyrin (hydrolyzed by nonspecific esterases, tributyrin esterase (22), and by aliesterases (23)). The relative rates

TABLE III

Cholinesterase activity in subcellular fractions of rat brain and beef pituitary

Tissue	Rat brain	Beef ant. pit.	Beef post. pit.
	Δ O.D./100 mg/10 minutes (acetylthiocholine)		
Homogenate	8.3 6.6 (7.2) 6.8	0.63 0.70 (0.67) 0.67	1.48 1.55 (1.50) 1.48
Total recovered activity	8.5 7.9 (8.2) 8.1	0.77 0.74 (0.77) 0.80	1.77 1.74 (1.76) 1.77
	Percentage of total recovered activity		
Nuclei	5.9 5.7 (5.6) 5.1	14.7 17.9 (16.0) 15.3	10.1 10.9 (10.3) 9.9
Secretory granules	—	7.5 8.8 (7.3) 5.6	5.8 6.0 (5.8) 5.7
Mitochondria	5.9 6.4 (5.9) 5.5	19.0 19.9 (19.2) 18.8	14.1 15.1 (14.1) 13.0
Microsomes	49.0 46.9 (48.3) 49.1	14.7 13.2 (14.6) 15.9	14.1 14.2 (14.0) 13.9
Supernatant	39.2 41.0 (40.2) 40.3	44.1 40.2 (42.9) 44.4	55.9 53.8 (55.8) 57.5

NOTE: Figures in parentheses are averages.

of hydrolysis of these substrates and the degree of inhibition by eserine are shown in Table IV. The microsome and supernatant fractions catalyzed the hydrolysis of each of the substrates at comparable rates, with the exception of tributyrin, which appeared to be hydrolyzed more rapidly by the microsome than by the supernatant fraction.

The highest rate of hydrolysis occurred with acetylcholine as the substrate. Acetyl-β-methylcholine was hydrolyzed well, but there was little hydrolysis of benzoylcholine. These results indicate the presence of a preponderance of true cholinesterase and the absence of pseudocholinesterase. All of these fractions hydrolyzed tributyrin to a considerable extent. Eserine 1×10^{-5} *M* produced 91 to 100% inhibition of the hydrolysis of acetylcholine, acetyl-β-methylcholine, and benzoylcholine, but inhibited tributyrin hydrolysis by only 10 to 19%. Thus the enzyme(s) responsible for the hydrolysis of tributyrin cannot be responsible for any major part of the hydrolysis of acetylcholine, acetyl-β-methylcholine, or benzoylcholine. Increasing the concentration of eserine to 3×10^{-5} *M* produced not more than 37% inhibition of tributyrin hydrolysis. This suggests that the hydrolysis of tributyrin in these fractions may be due to simple esterases.

The cholinesterases present in the fractions of beef anterior and posterior pituitary were characterized and compared to those found in rat brain on the

TABLE IV

Substrate specificity and eserine sensitivity of cholinesterases in subcellular fractions of rat brain

Fraction	Acetylcholine				Acetyl-β-methylcholine activity*		Benzoylcholine activity*		Tributyrin				
	Activity		% inhibition by eserine 1×10^{-5} M						Activity		% inhibition by eserine		
											1×10^{-5} M		3×10^{-5} M	
Homogenate	524		90		278		32		328		9		19	
	530	(527)	91	(92)	300	(280)	38	(37)	340	(334)	9	(10)	18	(19)
	526		94		261		31		333		12		19	
Microsomes	211		95		120		14		154		19		37	
	214	(216)	96	(94)	125	(121)	14	(15)	150	(155)	18	(19)	36	(37)
	222		92		118		17		162		21		37	
Supernatant	200		90		110		15		91		17		37	
	202	(204)	89	(91)	114	(109)	18	(15)	100	(93)	18	(17)	36	(35)
	210		93		102		12		89		17		33	

NOTE: Activity is expressed as μl CO_2 evolved/100 mg/30 minutes. Figures in parentheses are averages.
*Inhibited 100% by 1×10^{-4} M eserine.

TABLE V

Ratios of acetylthiocholine to butyrylthiocholine hydrolysis and eserine sensitivity of cholinesterases in various subcellular fractions of rat brain and beef pituitary

Fraction	Rat brain				Beef ant. pit.				Beef post. pit.			
	Ratio		Eserine* sensitivity		Ratio		Eserine sensitivity		Ratio		Eserine sensitivity	
Homogenate	9.6		86.3		1.0		36.1		1.1		64.8	
	9.3	(9.7)	88.0	(88)	0.9	(1.0)	37.4	(36)	1.1	(1.1)	68.8	(67)
	10.2		89.6		1.1		35.6		1.2		65.9	
Nuclei	9.2		96.0		1.3		26.0		0.9		56.8	
	8.8	(8.6)	97.5	(95)	1.3	(1.3)	27.8	(25)	0.9	(0.9)	55.1	(57)
	7.9		91.6		1.4		21.9		1.0		59.9	
Secretory granules	—		—		1.4		0		1.0		48.9	
					1.2	(1.4)	0	(0)	1.1	(1.0)	50.5	(50)
					1.6		0		1.0		50.7	
Mitochondria	8.5		87.4		0.8		38.0		1.0		86.2	
	8.6	(8.4)	88.8	(89)	0.7	(0.8)	40.4	(40)	0.9	(1.0)	83.9	(86)
	8.2		89.3		1.0		41.5		1.1		89.0	
Microsomes	8.6		88.0		1.2		48.5		1.2		68.1	
	8.4	(8.4)	90.0	(87)	1.4	(1.3)	46.5	(50)	1.1	(1.1)	67.0	(67)
	8.3		83.0		1.3		55.1		1.1		66.0	
Supernatant	10.5		90.1		2.1		43.0		1.3		60.9	
	10.8	(10.8)	87.4	(88)	1.8	(2.0)	44.5	(44)	1.1	(1.2)	62.2	(62)
	11.1		86.6		2.2		43.6		1.3		62.8	

NOTE: Figures in parentheses are averages.
*Percentage of inhibition of acetylthiocholine hydrolysis by 1×10^{-4} *M* eserine.

basis of the relative rates of hydrolysis of acetylthiocholine and butyrylthiocholine. The ratios of these activities are shown in Table V. The cholinesterase of brain fractions hydrolyzed acetylthiocholine 8.4 and 10.8 times as rapidly as they did butyrylthiocholine, whereas the ratio was between 0.8 and 2.0 for all fractions of both lobes of the pituitary. This indicates that brain contains a much higher percentage of acetylcholinesterase or true cholinesterase than either lobe of the pituitary. Brain and pituitary fractions exhibited similar, relatively low, activity against butyrylthiocholine. Table V also shows the inhibition of acetylthiocholine hydrolysis by eserine (1×10^{-5} *M*) in the various fractions. The degree of inhibition was high in the brain fractions, low in all anterior pituitary fractions, and intermediate in the posterior pituitary fractions.

Discussion

In our experiments approximately 50% of the cholinesterase activity, as measured by the hydrolysis of acetylcholine or acetylthiocholine, was present in the microsome fraction of rat brain. This is slightly higher than, but in general agreement with, the percentages found in brain microsomes by other workers (11, 12, 13, 14). In our experiments, high activity also was observed in the supernatant fraction, although the previous reports indicated that this fraction contained only 8% (11), 15% (12), or 25% (calculated from data published by Holmstedt and Toschi (13)) of the total. Aldridge (12) reported the total activity of all the fractions to be approximately 100% of the homogenate activity, whereas we consistently found the sum of the activities of the fractions to be higher than that of the homogenate. The high recoveries obtained in our experiments may partially explain why we found the activity in the supernatant fraction to be higher than that reported by previous authors. In addition, we observed rather lower activity in the mitochondria fraction than did previous investigators, and it is possible that part of the activity in our supernatant fraction was due to mitochondrial enzyme removed during washing. Our experiments using homogenizing chambers with various clearances and different rotor speeds indicate that these factors do not influence the distribution of cholinesterases in the subcellular fractions obtained.

In rat brain microsome fractions, the high rates of hydrolysis of acetylcholine and acetyl-β-methylcholine, the low rates of hydrolysis of butyrylthiocholine and benzoylcholine, and the eserine sensitivity of this hydrolysis indicate that the enzyme involved is mainly true cholinesterase. This is in agreement with previous reports (12, 13). The enzyme in the supernatant fraction also appeared to be mainly true cholinesterase, splitting acetylthiocholine approximately 10 times as rapidly as it did butyrylthiocholine. This is in disagreement with the work of Holmstedt and Toschi (13), who reported ratios of acetylcholine to butyrylcholine hydrolysis of 5.6, 5.7, and 3.4. They interpreted their results to indicate relatively more pseudocholinesterase in supernatant than in microsome fractions of rat brain. These authors do not report the percentage of the total homogenate activity recovered in the fractions. In the present experi-

ments high recoveries were obtained, and if these were higher than those of Holmstedt and Toschi, this might explain the discrepancy in the ratios obtained. Aldridge (12) reported almost equal amounts of pseudocholinesterase to be present in microsome and supernatant fractions.

One may question the comparison of brain from one species with pituitary from another. However, brain cholinesterase has been found to be similar in all mammals which have been studied to date, including the bear and elephant (1), and it is unlikely that the cholinesterase in rat and beef brains differs significantly. Thus the cholinesterase activity of rat brain probably can be compared legitimately to that of beef pituitary gland, and it is very unlikely that differences noted between the two are due to species differences.

In contrast to brain, the cholinesterase in beef anterior and posterior pituitary was not localized in the microsome fraction, but was distributed throughout all of the subcellular fractions studied, with about 50% in the supernatant. Again in contrast to brain, the cholinesterase from both lobes of the pituitary split acetylthiocholine and butyrylthiocholine at similar rates. Since we found low but similar values for the hydrolysis of butyrylthiocholine in all three tissues, it can be concluded that there is a relative deficiency of true cholinesterase in beef anterior and posterior pituitary. In this respect posterior pituitary resembles anterior pituitary rather than brain, although its content of true cholinesterase is appreciably higher than that of the latter. In a recent histochemical study, Koelle and Geesey (24) found a similar differential in true cholinesterase content of the two lobes of the pituitary. Burgen and Chipman (25) also found relatively small amounts of true cholinesterase in the posterior pituitary of the dog.

The posterior pituitary is intermediate between brain and anterior pituitary with respect to both total cholinesterase content and degree of inhibition of acetylthiocholine hydrolysis by eserine. However, with respect to the total activity, nature, and distribution of cholinesterase, the posterior pituitary, which has a secretory function, more closely resembles the adjacent, secretory anterior pituitary than it does the brain from which it was derived during embryological development.

Acknowledgment

The authors wish to express their appreciation to W. L. Henry, D.V.M., Department of Agriculture, Winnipeg, for the supply of beef pituitary glands.

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MARINE STEROLS

IX. BIOSYNTHESIS OF 24-METHYLENECHOLESTEROL IN CLAMS[1]

U. H. M. FAGERLUND AND D. R. IDLER

Abstract

Clams (*Saxidomus giganteus*) are able to transform injected cholesterol-4-C^{14} into radioactive 24-methylenecholesterol in vivo. The highest rate of conversion was observed when the radioactive sterol was injected into the digestive gland. The findings suggest a possible pathway in the biosynthesis of C_{28}-sterols and throw some light on the question of the origin of C_{28}-sterols in lamellibranchs.

Introduction

Previous experiments (1) conducted in this laboratory have shown that two members of the phylum Mollusca, a mussel (*Mytilus californianus*) and a clam (*Saxidomus giganteus*), are able to synthesize a sterol from injected acetate-2-C^{14}. The chromatographic properties of the sterol are consistent with those of a monounsaturated Δ^5-sterol. Only a trace of radioactivity was found in the chromatographic zone containing 24-methylenecholesterol. This observation is significant in view of the fact that 24-methylenecholesterol constitutes more than 50% of the total sterols in clams. It would seem that either the turnover of 24-methylenecholesterol is very slow or this sterol is of exogenous origin. The present investigation reveals that injected cholesterol-4-C^{14} is converted to radioactive 24-methylenecholesterol in the clam. Thus it appears that at least part of the 24-methylenecholesterol found in clams is supplied by biosynthesis within the animal.

Experimental

Radioactivity was determined with a gas-flow counter equipped with a "micromil" window and specially shielded for low background counting.* The background count obtained with this instrument was of the order of 2 c.p.m. Melting points were determined either with a conventional melting point apparatus or on a Kofler block.

Procedure for Chromatography of Sterols and Steryl Acetates on Paper

A method for the chromatographic separation of sterols and steryl acetates on paper has been described by deZotti *et al.* (2). This method was adopted with slight modifications. Commercial kerosene was distilled and the fraction boiling at 220–240° collected. Whatman No. 1 chromatographic filter paper was impregnated with this fraction by dipping it into a 12% solution of kerosene in dichloromethane. Dichloromethane was evaporated by letting the paper hang in air a few minutes. In this way impregnation of approximately 150 mg/dm^2

[1]Manuscript received January 5, 1961.
Contribution from the Fisheries Research Board of Canada, Technological Station, Chemistry Section, Vancouver 8, B.C.

*Supplied by Nuclear-Chicago Corp., Chicago 10, Ill., U.S.A.

was obtained. The material to be chromatographed was then applied as quickly as possible to a line 8 cm from one end of the paper strip. Sterols were run as 20 μg spots and steryl acetates as 50 μg spots. Larger amounts were applied to the origin as a strip the length of which varied with the amount of compound.

Chromatographic jars were equilibrated with the mobile phase for 24 hours previous to chromatography. Sterol chromatograms were developed with 85% aqueous pyridine by ascending chromatography at 20° for 24–40 hours. Steryl acetate chromatograms were developed with 98% aqueous acetic acid by ascending chromatography at 25° for 24–48 hours.

Eighty-six hours was required for the solvent front to reach the top of a 55-cm paper strip. However, after this time, the spots were often diffuse. Both sterols and steryl acetates were sometimes developed by descending chromatography. The time of development was then much shorter.

After evaporation of the mobile phase the chromatograms were sprayed with a saturated solution of $SbCl_3$ in a 1:1 mixture of acetic acid and chloroform. They were heated in a drying cabinet at 80–90° for 5–10 minutes. Strips were located by cutting off and spraying a small portion of the chromatogram containing some of the material on each side of the strip. Standards were run on each side of a strip.

When chromatographed as described, ketosteryl acetates run with a speed more than twice that of the corresponding monohydroxysteryl acetates. Table I shows the separation obtained with certain pairs of steryl acetates.

TABLE I

Approximate distance between centers of spots after chromatography of steryl acetates on paper

	24 hours	48 hours
24-Ketocholesterol—25-ketocholesterol	2.3 cm	3.1 cm
24-Methylenecholesterol—25-dehydrocholesterol	1.5 cm	1.5–2.1 cm
Sitosterol—cholesterol	1.3 cm	

The acetate with the smaller molecular weight is faster-running. Cholesteryl acetate is slower than either 24-methylenecholesteryl acetate or 25-dehydrocholesteryl acetate. A somewhat better separation was obtained with free sterols.

20-Dehydrocholesteryl acetate and its ozonolysis product, 20-keto-21-norcholesteryl acetate, have nearly the same mobility as 24-methylenecholesterol acetate and 24-ketocholesteryl acetate, respectively, and a separation was not possible.

Experiment I

Injection of Cholesterol-4-C^{14} and Isolation of 24-Methylenecholesterol

Clams were kept during the month of July in an aquarium supplied with running, aerated sea water. Cholesterol-4-C^{14},* with a specific activity (S.A.)

*Supplied by Beta Laboratories Inc., Philadelphia, Pa., U.S.A.

of 4.05×10^6 c.p.m./mg, was dissolved in 0.2 ml of salmon oil, which was injected at random into two clams with a 22-gauge hypodermic needle forced in between the closed shells. No attempt was made to establish the amount of cholesterol-4-C^{14} actually received by the tissues of the clams, since losses which are difficult to determine quantitatively occur during the injection. One week after the injection, the soft parts of the animals were macerated in a Servall Omni-Mixer and extracted three times with hot acetone. The solution was concentrated to an oil (2.8×10^7 c.p.m.) which was saponified with potassium hydroxide. The non-saponifiable portion was extracted from the saponification mixture in the usual manner and was then chromatographed on neutral aluminum oxide "Woelm"† (activity grade II). The sterol fraction was eluted with a 1:1 mixture of ethanol and dichloromethane. The *p*-phenylazobenzoyl esters of the sterol mixture were prepared (3) and chromatographed on silicic acid – Celite 535 using petroleum ether-benzene (5.5:1) to develop the chromatogram (4). Two colored zones developed. The lower zone contained unchanged cholesterol-4-C^{14}. The upper zone, which, according to our previous experiments (5), contained 24-methylenecholesterol, was eluted. This zone was contaminated with some cholesteryl azoate of high specific activity probably caused by surface spreading (4) of the lower zone. In order to remove most of this material the upper zone azoate was chromatographed together with carrier cholesteryl azoate. The upper zone was again eluted and the same procedure was repeated once more. The 24-methylenecholesteryl azoate was finally crystallized from benzene – aqueous methanol to remove impurities originating from the adsorbent, and hydrolyzed. The free sterol weighed 57 mg and had a radioactivity of 44,550 c.p.m. or a S.A. of 782 c.p.m./mg. The major portion of the radioactivity found in the non-saponifiable matter was recovered from the combined cholesteryl azoate zones (2.3×10^7 c.p.m.).

Radioactive 24-methylenecholesterol (9.8 mg) obtained through hydrolysis of the upper zone azoate was mixed with 44.1 mg of authentic 24-methylenecholesterol previously isolated from clams and the mixture was acetylated. The acetate was crystallized 8 times from aqueous methanol. The S.A. diminished from 129 c.p.m./mg to 39.6 c.p.m./mg without reaching a constant value.

Supernatants from the above crystallizations were combined and the combined material crystallized twice from aqueous ethanol. The S.A. was then 66 c.p.m./mg. The material (1.11 mg) was chromatographed on paper as a 10-cm long strip for 24 hours. After the zone had been located in the usual manner it was eluted and rechromatographed for 66 hours. The S.A. of the twice chromatographed acetate was 30.3 c.p.m./mg.

Ozonolysis of Upper Zone Acetate and Isolation of 24-Ketocholesteryl Acetate

Radioactive 24-methylenecholesterol (15 mg) from the upper zone of the azoate chromatogram was combined with 76 mg of authentic 24-methylenecholesterol obtained from clams. The mixture was acetylated. The acetate

†Supplied by Alupharm Chemicals, P.O. Box 755, New Orleans, La., U.S.A.

was suspended in a 4:1 mixture of acetic anhydride and acetic acid and ozonized in an ice–salt bath. During the ozonolysis most of the acetate dissolved in the reaction mixture. Zinc dust and water were added to the solution and the ozonides decomposed by boiling for 20 minutes. After the solution had cooled an excess of water was added and steryl acetates were recovered by extraction with ether, washing with $NaHCO_3$, and evaporation of the ether. The acetate mixture was chromatographed on aluminum oxide of activity grade II. Unreacted steryl acetate was eluted with heptane–benzene (1:1). The ketonic material was eluted in two fractions with benzene–dichloromethane (1:1).

Fraction 1 had a radioactivity of 488 c.p.m. It was crystallized 4 times from aqueous methanol. A slight change in S.A. occurred in the first crystallization, but subsequent values remained constant, viz. 26.5 c.p.m./mg.

Fraction 2 of the ketonic material obtained by chromatography of the ozonolysis products had a radioactivity of 914 c.p.m., but the weight of the crude material left after evaporation of the solvent was only 3 mg. One half of this fraction was applied as a strip to a paper chromatogram and chromatographed for 23 hours. The major part of the $SbCl_3$ positive material had travelled the same distance as the 24-ketocholesteryl acetate standard, namely 17.7 cm from the origin. There was some material in the 25-ketonorcholesteryl acetate section and part of the substance had travelled ahead of 25-ketonorcholesteryl acetate. Four sections were cut out, eluted, and counted: section 1 (27.5 c.p.m., 2 cm wide) with the motility of 24-ketocholesteryl acetate, section 2 (96.6 c.p.m., 2 cm wide) with that of 25-ketonorcholesteryl acetate, section 3 (36.5 c.p.m., 2.3 cm wide) immediately ahead of the latter, and section 4 (12.0 c.p.m., 3.0 cm wide) immediately behind 24-ketocholesteryl acetate. The material from Sections 1 and 4, was rinsed off the planchets, combined, and rechromatographed as an 8 cm long strip for 24 hours. When the chromatogram was dried, and the various sections cut out and eluted, the 24-ketocholesteryl acetate section (2 cm wide) carried 11.6 c.p.m., the 25-ketonorcholesteryl acetate section (2 cm wide) carried 6.8 c.p.m., and the section immediately behind 24-ketocholesteryl acetate (2 cm wide) carried 4.8 c.p.m. The S.A. of 24-ketocholesteryl acetate from this chromatogram was 27.2 c.p.m./mg.

Separation of 20-Keto-21-norcholesteryl Acetate from 24-Ketocholesteryl Acetate by Crystallization

A mixture of 1.8 mg of 20-keto-21-norcholesteryl acetate (plates, m.p. 147°) and 4.35 mg of 24-ketocholesteryl acetate (needles, m.p. 129°) was crystallized from aqueous methanol. Melting points recorded were: I, 105–120°; II, 120–124°; III, 127°; V, 128°. The acetates of later crystallizations formed needles. The melting point was markedly depressed when the material was mixed with 20-keto-21-norcholesteryl acetate. A gas chromatographic analysis of the final product (V) revealed it to be 24-ketocholesteryl acetate, which contained less than 4% impurities.

The remaining half of fraction 2 from the column chromatography of the

ozonolysis products of radioactive 24-methylenecholesteryl acetate was mixed with 2.65 mg of 20-keto-21-norcholesteryl acetate. The mixture (m.p. 105–110°) was crystallized 8 times from aqueous methanol. The S.A. of the crystallized material (needles, m.p. 135–137°) was only 1.4 c.p.m./mg. Gas chromatographic analysis was made of a sample obtained by pooling the supernatants from the last three crystallizations and crystallizing the pooled material once. According to the analysis, this sample was 89% pure 20-keto-21-norcholesteryl acetate containing 8% of 24-ketocholesteryl acetate.

Experiment II

Injection of Cholesterol-4-C^{14} and Isolation of 24-Methylenecholesterol

Five clams, 2–3 years of age, taken in the month of February were kept in seawater (aerated, but not running) at 17°. Cholesterol-4-C^{14}* (S.A., 8.25×10^7 c.p.m./mg) was dissolved in a few drops of olive oil. The oil was injected into the digestive glands by inserting the needle between the closed shells close to the hinge. The animals apparently did not suffer any ill effects from the injections. Four clams (batch 1) were kept for 4–9 days in an aquarium, and one clam (batch 2) was kept for 11 days. The water in the aquarium was changed once, on the third day after the injection.

The procedure for extraction of the oil and for obtaining the non-saponifiable matter has been described in experiment I. There were 7.39×10^6 c.p.m. in the oil from batch 1. 24-Methylenecholesterol from this batch was obtained by chromatography of the non-saponifiable matter (6.83×10^6 c.p.m.) on paper without preliminary purification. Approximately 1.3 mg (4230 c.p.m.) was chromatographed in the 85% pyridine chromatographic system. The section containing 24-methylenecholesterol (259 c.p.m.) was cut out. The sterol was eluted and rechromatographed in the same system. 24-Methylenecholesterol obtained from this chromatogram weighed 0.142 mg (86.7 c.p.m.). The material was mixed with 1.265 mg of authentic 24-methylenecholesterol and crystallized twice from aqueous methanol. The S.A. dropped sharply in the first crystallization, but changed only slightly in the second. The diluted material was finally chromatographed on paper, and the compound obtained from the 24-methylenecholesterol area was crystallized once. The S.A. was slightly higher, 20.6 c.p.m./mg.

There were 2.36×10^6 c.p.m. in the oil fraction from batch 2. To isolate the sterols, the non-saponifiable portion (1.48×10^6 c.p.m.) was chromatographed on alumina of activity grade II as described in experiment I. The sterol fraction weighed 33.7 mg (1.29×10^7 c.p.m.).

This fraction was ozonized and resulting ketosteryl acetates were isolated as described above in experiment I. They were chromatographed on paper in the 98% HAc system. The 24-ketocholesteryl acetate section was cut out and the acetate eluted and rechromatographed in the same system. Thus 0.461 mg (1150 c.p.m./mg) of 24-ketocholesteryl acetate was obtained. The material

*Supplied by Merck & Co. Ltd., Montreal, Que.

was mixed with authentic 24-ketocholesteryl acetate (3.163 mg) and the mixture was crystallized repeatedly from aqueous methanol. After an initial sharp drop in the first crystallization, the S.A. remained constant at 38.5 c.p.m./mg. On the basis of this value, the S.A. of the original 24-methylenecholesterol was calculated to be 336 c.p.m./mg.

Results

Experiment I

Cholesterol-4-C^{14} was injected at random into clams, which were held in an aquarium for 7 days. After extraction of the clams, saponification of the oil, and chromatography of the non-saponifiable matter, 24-methylenecholesterol was isolated from the sterol mixture through chromatography of the azoyl esters. The free sterol was acetylated and mixed with carrier 24-methylenecholesteryl acetate and the mixture was crystallized several times. 24-Methylenecholesteryl acetate was separated from any 25-dehydrocholesteryl acetate present and from residual cholesteryl acetate by chromatographing the crystallization product twice on paper. The S.A. of 24-methylenecholesterol acetate was then 30.3 c.p.m./mg. The true S.A. of the original 24-methylenecholesterol before dilution was calculated on the basis of this value and was found to be 184 c.p.m./mg.

Another sample of 24-methylenecholesterol obtained from the azoyl ester chromatogram was mixed with carrier 24-methylenecholesterol; the mixture was acetylated, and ozonized. After splitting of the ozonides, crude 24-ketocholesteryl acetate was separated from unreacted material on alumina. A portion of the 24-ketocholesteryl acetate fraction (fraction 1) was crystallized to a constant S.A. of 26.5 c.p.m./mg. The S.A. of the original undiluted 24-methylenecholesterol calculated on the basis of this value would be 179 c.p.m./mg.

Another portion (fraction 2) of the ozonolysis product was purified by chromatography on paper. After being chromatographed twice, the 24-ketocholesteryl acetate had a S.A. of 27.2 c.p.m./mg or the same as obtained by crystallization. Fraction 2 originally had a much higher S.A. The major part of the radioactivity had the same mobility on the first paper chromatogram as 25-ketonorcholesteryl acetate. The extent of conversion of cholesterol-4-C^{14} to 24-methylenecholesterol was calculated on the basis of the average S.A. (26.9 c.p.m./mg) of 24-ketocholesteryl acetate. Taking into account a 24-methylenecholesterol content of approximately 80 mg in the two clams, the radioactivity in 24-methylenecholesterol was 0.052% of the total radioactivity extracted from the animals with acetone.

Experiment II

Cholesterol-4-C^{14} was injected into the digestive glands of five clams. The clams used in this experiment were younger than the ones in the previous experiment. Four clams (batch 1) were kept from 4 to 9 days. One clam (batch 2) was kept for 11 days before being extracted.

24-Methylenecholesterol was isolated from the non-saponifiable matter obtained from batch 1 by chromatography on paper. After the sterol had been chromatographed on paper once more it was mixed with carrier 24-methylenecholesterol and crystallized to a constant S.A. of 18.9 c.p.m./mg. The diluted material was chromatographed on paper and thereafter crystallized once. The S.A. was 20.6 c.p.m./mg. The S.A. of original, undiluted 24-methylenecholesterol calculated on the basis of this value was 204 c.p.m./mg. There was 87 mg of 24-methylenecholesterol present in the acetone extract of this batch as determined by chromatographic separation of the azoyl esters. The radioactivity carried by 24-methylenecholesterol represents 0.25% of the total radioactivity extracted from the animals with acetone.

The total sterol fraction from batch 2 was acetylated, ozonized, and the ozonides decomposed. Ketosteryl acetates were separated from unreacted material by chromatography of the ozonolysis products on alumina and were twice chromatographed on paper. 24-Ketocholesteryl acetate, thus obtained, was mixed with authentic 24-ketocholesteryl acetate and crystallized to a constant specific activity of 38.5 c.p.m./mg. The S.A. of original, undiluted 24-methylenecholesterol determined on the basis of this value was 336 c.p.m./mg. There was 34 mg of sterols in this batch. Assuming that the ratio of the amount of cholesterol to that of 24-methylenecholesterol is the same as in batch 1, the radioactivity carried by 24-methylenecholesterol from this batch represents 0.30% of the total radioactivity extracted from the animal with acetone.

The only known sterols which might possess chromatographic and solubility properties similar to those of 24-methylenecholesterol and which could be ozonized as acetates to steryl acetates with chromatographic and solubility properties similar to those of 24-ketocholesteryl acetate are 25-dehydrocholesterol, 20-dehydrocholesterol, and fucosterol (24-ethylidene cholesterol). Previous work (6) in this laboratory has shown that, when radioactive cholesterol is injected into clams, a radioactive C-25 unsaturated sterol, believed to be 25-dehydrocholesterol, accompanies 24-methylenecholesterol when the azoates are chromatographed. In the present investigation the high radioactive count in fraction 2, experiment I, from the alumina chromatogram of the ozonolysis products indicates the presence in the sterol fraction of 25-dehydrocholesterol carrying a higher S.A. than that of 24-methylenecholesterol. To exclude contamination assignable to this source, samples of radioactive 24-methylenecholesteryl acetate and its ozonolysis product have been chromatographed on paper in systems that separate these compounds from the corresponding 25-dehydrocholesterol derivatives.

The second possible contaminant, 20-dehydrocholesteryl acetate, did not separate effectively from 24-methylenecholesteryl acetate by the paper chromatographic method used in this investigation. This is the case also in regard to the corresponding ketosteryl acetates. However, it is possible to separate the ozonolysis product, 20-keto-21-norcholesteryl acetate, from 24-ketocholesteryl

acetate by crystallization. When a mixture of 1.8 mg of the former and 4.35 mg of the latter was crystallized 5 times from aqueous methanol, the latter was obtained reasonably free from 20-keto-21-norcholesteryl acetate as evidenced by melting point, mixed melting point, and gas chromatographic analysis. Since it was possible to crystallize the ozonolysis product of 24-methylenecholesteryl acetate to a constant S.A. it follows that the radioactivity is not attributable to 20-keto-21-norcholesteryl acetate.

Additional evidence for the absence of any appreciable amount of radioactive 20-keto-21-norcholesteryl acetate in the radioactive 24-ketocholesteryl acetate was obtained by crystallization of a small amount of radioactive 24-ketocholesteryl acetate (fraction 2) mixed with a larger amount of 20-keto-21-norcholesteryl acetate. It is possible to separate the two compounds by crystallization even when they are present in this proportion as evidenced by melting point and gas chromatographic analysis. If the radioactivity attributed to 24-ketocholesteryl acetate had, in fact, been caused by the presence of a minor amount of radioactive 20-keto-21-norcholesteryl acetate, then the sample should have retained this activity. From the results of a chromatographic analysis of fraction 2, it was estimated that the material mixed with 20-keto-21-norcholesteryl acetate contained more than 1 mg of 24-ketocholesteryl acetate. If the radioactivity in this sample (>27.2 c.p.m.) was attributable to 20-keto-21-norcholesteryl acetate, then the final crystallization product (20-keto-21-norcholesteryl acetate) when free from 24-ketocholesteryl acetate would have had a S.A. greater than 27.2 c.p.m./2.65 mg or greater than 10.3 c.p.m./mg. However, the S.A. diminished to a value of 1.4 c.p.m./mg through eight crystallizations.

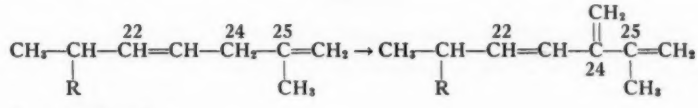
The third possible contaminant of the radioactive 24-methylenecholesterol, fucosterol, is ruled out because of the difference in chromatographic properties. In the 85% acetic acid system, fucosteryl acetate is well separated from the faster-running 24-methylenecholesteryl acetate.

Discussion

Carbon-28 in the molecule of sterols and triterpenes is known to be derived from one-carbon donors, such as formate, serine, and methionine (7, 8, 9). However, little information is available relating to synthetic pathways. The present work indicates that the addition of carbon-28 might be one of the last steps in the biochemical sequence leading to C_{28}-sterols. In the biosynthesis of these sterols from acetate, it appears that cholesterol is an earlier product. This theory may offer an explanation to one of our earlier observations: When clams were injected with acetate-2-C^{14} the major part of the radioactivity, subsequently found in the non-saponifiable fraction, resided in a sterol presumed to be cholesterol, but very little activity was found in the 24-methylenecholesterol fraction. Possibly another explanation is provided by Clark and Bloch's concept (10) of a differentiation in sterols of the hide beetle into two functional types, viz. structural and metabolic, each with an implied different

rate of turnover.

Tschesche and Korte (11) have advanced a theory concerning the mode of formation of C_{28}- and C_{29}-sterols in plants. According to these authors, the activation of C-24 by a system consisting of a C-22 and a C-25 double bond (Fig. 1) would facilitate the addition of one or more carbon units at C-24.



R = sterol nucleus

FIG. 1.

Initially a C-22,24(28),25 triunsaturated sterol would conceivably be formed which, by the saturation of appropriate double bonds, would give rise to all known C_{28}- and C_{29}-sterols. If the theory is applied to the synthesis of sterols in clams, saturation of the C-22 and C-25 double bonds would result in the formation of 24-methylenecholesterol. The observations that radioactive cholesterol can be converted by the same animal into C-22 and C-25 unsaturated C_{27}-sterols (6), and into a C-24 (28) unsaturated C_{28}-sterol, together give support to this theory.

Acknowledgments

We are greatly indebted to Dr. Franz Sondheimer, The Weizmann Institute of Science, Rehovoth, Israel, for supplying us with a sample of 21-nor-20-ketocholesteryl acetate and 20-dehydrocholesteryl acetate; to Dr. C. L. Sweeley, University of Pittsburg, for carrying out the gas chromatographic analysis; to Dr. D. B. Quayle, Fisheries Research Board of Canada, Biological Station, Nanaimo, B.C., for supplying the clams; and to Dr. M. Newman, Curator, Vancouver Public Aquarium, for supplying facilities for holding the clams in captivity.

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THE ACYLATION OF MONOACYL-L-α-GLYCERYLPHOSPHORYLCHOLINE UNDER VARIOUS CONDITIONS[1]

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Abstract

Monoacyl-L-α-GPC's (lysolecithins) were converted to their cadmium chloride complexes and acylated with myristoyl chloride in chloroform in the presence and in the absence of pyridine. Both preparations of the diacyl-L-α-GPC's were reacted with phospholipase A of *Crotalus adamanteus* venom, and the nature of the fatty acids released, as well as the lysolecithin fatty acids, was determined by gas-liquid chromatography. The compositions of the fatty acids were different in the two preparations. These findings could indicate that acyl migration occurs during the acylation of the lysolecithin cadmium chloride complexes both in the presence and absence of pyridine, or that phospholipase A does not invariably remove the acyl group attached to the β-position in lecithin. The nature and possible causes of these variations are discussed.

Introduction

Several papers dealing with the action of phospholipase A on natural, synthetic, and semisynthetic lecithins have been published during recent years. The conclusions reached in these papers are not uniform and sometimes they do not appear to be fully justified by the data presented. The present paper, therefore, presents a brief review of the literature together with data on the composition of a semisynthetic lecithin. A subsequent paper will present a mathematical analysis of some published and new data on the site of action of phospholipase A.

In 1955, Tattrie and McArthur (1) prepared L-α-glycerylphosphorylcholine (L-α-GPC) by hydrolysis of egg lecithins with 5% mercuric chloride in 50% ethanol. The same authors (2) reported the acylation of L-α-GPC to a diacyl-L-α-GPC with palmitoyl chloride in a medium of chloroform.

Baer and Buchnea (3) later prepared L-α-GPC by (*a*) the method of Tattrie and McArthur, (*b*) the method of Dawson (4), which uses dilute alkali for hydrolysis of egg lecithin, and (*c*) a synthesis *de novo* by the method of Baer and Kates (5). They converted these samples of L-α-GPC to the cadmium chloride complexes and acylated them with acid chlorides in pyridine to yield diacyl-L-α-GPC. From measurements of the specific rotation of the three samples of diacyl-L-α-GPC, they concluded that some racemization had occurred during the preparation of L-α-GPC by the method of Tattrie and McArthur, and also by the method of Dawson.

The first reported synthesis of mixed L-α-lecithins is that of Levene, Rolf, and Simms (6), who acylated lysolecithins with acid anhydrides. Since 1959,

[1]Manuscript received March 20, 1961.
Contribution from the Division of Applied Biology, National Research Council, Ottawa, Canada.
Issued as N.R.C. No. 6407.
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the route suggested by Baer and Buchnea (3) for the synthesis of "mixed acid" L-α-lecithins has been followed by numerous authors. Bennett and Tattrie (7) reported a synthesis of a mixture of palmitoyl–myristoyl and stearoyl–myristoyl L-α-GPC obtained by the acylation of the cadmium chloride complexes of monopalmitoyl and monostearoyl L-α-GPC with myristoyl chloride in chloroform. Hanahan, Brockerhoff, and Barron (8) reported a similar synthesis in which the acylation was carried out in pyridine. Subsequently, Hanahan and Brockerhoff (9) reported the synthesis of palmitoyl–oleoyl-L-α-GPC's by the acylation of the cadmium chloride complex of monopalmitoyl-L-α-GPC with oleoyl chloride in a medium of pyridine. Later, a further paper (10) appeared which was partially based on this synthesis. De Haas *et al.* published three papers in 1960 (11, 12, 13) relating to the synthesis of diacyl-L-α-GPC's. They described a synthesis *de novo* of mixed diacyl-α-GPC's (11) and a partial synthesis of "mixed acid" L-α-lecithins. This latter synthesis was carried out essentially by the method used by Hanahan and Brockerhoff (9).

Some of the abovementioned papers also discuss the site of action of phospholipase A on these synthetic lecithins. Although Latzer (14) pointed out in 1927 that lysolecithin does not necessarily contain only saturated fatty acids, and Ogawa (15) found in 1936 that a hemolytic product could be obtained by the action of a pancreatic lecithinase A on hydrogenated egg lecithin, it was, until 1952, generally assumed that the phospholipase A of snake venoms and commercial pancreatin removed the unsaturated fatty acids from diacyl-L-α-GPC's. In 1952, Zeller (16) reported that this enzyme would react with diacyl-L-α-GPC's containing only saturated acyl groups and suggested that the enzyme was specific for acyl groups linked to a certain position in the diacyl-L-α-GPC's rather than for unsaturated acids. Subsequently, Hanahan (17) and Long and Penny (18) reported two independent oxidative procedures for the elucidation of the structure of monoacyl-L-α-GPC. They interpreted their results as indicating that the phospholipase A of snake venoms removed the acyl groups attached to the α′-position of the diacyl-L-α-GPC. Later, Davidson, Long, and Penny (19), working along the lines suggested by Hanahan (17), reaffirmed the earlier conclusion that cleavage by the venom phospholipase A occurred at the α′-position in the lecithin.

After the site of action of phospholipase A was elucidated, numerous workers, accepting the proof offered for the positional attack of lecithinase A, used this enzyme to determine the distribution of fatty acids in lecithins obtained from various sources. Hanahan (20, 21) utilized the enzyme to determine the distribution of fatty acids in lecithins obtained from eggs, and the livers of cattle, rabbits, dogs, guinea pigs, and rats. Hanahan and Blomstrand (22) investigated the sites at which 1-C^{14}-labelled palmitic and oleic acids were incorporated into the lecithins of various organs of rats, while Rhodes and Lea (23) and Rhodes (24) used the enzyme to study the distribution of fatty acids in the glycerol phosphatides of egg yolks. Other workers who have used

this enzyme are Debuch (25), with lecithins of soya beans, and Johnston and Nakayama (26), with lecithins obtained from bile.

The oxidative procedure first developed by Hanahan (17) has been used indirectly by Uziel and Hanahan (27) to illustrate the presence of a lysolecithin migratase in *Penicillum notatum*. Rimon and Shapiro (28) also used the oxidative procedure to elucidate the structure of lysophosphatidic acid. Gray (29, 30) used a similar procedure to illustrate the position of the acyl linkage in phosphatidal choline, and Rapport and Franzl (31) concluded that since phospholipase A reacts with phosphatidal choline at the same rate as it does with phosphatidyl choline, then the acyl group in phosphatidal choline must be on the α′-position. However, Marinetti and Erbland (32) concluded, from the results of an investigation of the structure of pig-heart phosphatidal choline by a method not involving oxidation, that the acyl group was in the β-position.

Marinetti and Temple (33), in a preliminary report, stated that when lysolecithins were hydrogenated and oxidized with buffered permanganate or bromine water, two lyso compounds were formed, one being a keto derivative. In a more detailed paper, Marinetti, Erbland, and Stotz (34) concluded that the lysolecithins prepared from egg lecithins or rat liver lecithins by the action of snake venom were mixtures of α′-and β-monoacyl derivatives of L-α-GPC. However, Marinetti, Erbland, Temple, and Stotz (35) later reported that "the amount of keto lyso compound formed on oxidation of lysolecithin with buffered bromine water varies between 54% and 94%, depending upon the pH at which the oxidation is carried out," and that lysolecithinic acid (the compound that they would expect from a β-acyl-L-α-GPC) had not been found in the reaction products. They also claimed that passage of lysolecithin through a silicic acid column caused some migration of the acyl group from the β- to the α-position. Marinetti, Erbland, and Stotz (36) have also reported that phospholipase A of snake venom shows chain length preference for some lecithin fatty acids.

In 1959, Tattrie (37) reported, as a result of studies using phospholipase D, pancreatic lipase, and phospholipase A on egg lecithins, that the phospholipase A removed the fatty acids from the β-position in these lecithins. However, reservations were made because of the limited accuracy of the gas-liquid chromatographic apparatus used. Later, Hanahan *et al.* (8, 9, 10) confirmed these results and stated that phospholipase A reacts on lecithins by removing the β-linked acyl groups. These results were further confirmed on synthetic substrates by de Haas, Mulder, and van Deenen (38). However, Blomstrand (39), who used phospholipase A to demonstrate the position of fatty acids in human bile lipids, states that "the specificity of phospholipase A therefore seems well established, although further work is necessary to definitely settle the question about the site of attack of the enzyme."

From the above brief review it would appear that three principal methods have been followed for illustrating the site of action of this enzyme.

(*a*) Method I involves the oxidation, or oxidation followed by subsequent hydrolysis of the oxidation products formed from lysolecithin. It is generally agreed that this is not a completely satisfactory method since answers obtained by it conflict with answers obtained by other methods, and also different workers have reported different results although the methods used were essentially the same. Furthermore, the postulated reaction mechanism of Marinetti, Erbland, and Stotz (34), which embodies that of both Hanahan (17) and Gray (30), is based on the assumption that no hydrolysis of ester linkages occurs during oxidation. However, as Marinetti, Erbland, and Stotz (34) point out, this assumption may not be entirely valid.

(*b*) Method II involves the action of phospholipase D on lecithins, and the glyceride so formed is reacted with pancreatic lipase (either immediately or after converting it to a triglyceride) to determine the fatty acid on the α-position. The fatty acids released by phospholipase A are determined on the same lecithins. This pathway is of considerable value as, under certain circumstances, it could lead to the complete definition of a reaction sequence. However, it depends on the assumption that pancreatic lipase removes only the α-fatty acids from an α, β-diglyceride and the α, α'-fatty acids from a triglyceride.

(*c*) Method III involves the synthesis of "mixed acid" lecithins of known configuration followed by their reaction with phospholipase A. This is the ideal method but, regrettably, there is no method yet developed to prove that the compounds synthesized are the ones that the workers set out to synthesize.

The present paper deals with a process that was used in several of the previously mentioned researches involving the synthesis of "mixed acid" lecithins, but points out that a different interpretation may be placed on the results.

Methods and Results

Phosphorus, nitrogen, and choline analyses were carried out by the methods of King (40) and Dumas and Glick (41) respectively.

Fatty acids were determined as follows: the lipid sample was hydrolyzed in 2 *N* HCl under reflux for 2 hours; the fatty acids were extracted with ethyl ether, dried over anhydrous sodium sulphate, and concentrated to dryness in a stream of nitrogen. The fatty acids were dissolved in anhydrous ethyl ether and converted to the methyl esters with diazomethane. Individual fatty acids were determined by gas–liquid chromatography on a 4-foot column of Apiezon M, 5% on celite (British Drug Houses (80–120 mesh) washed with concentrated hydrochloric acid, distilled water, 10% sodium hydroxide, and distilled water) with a Pye Argon Chromatograph (Technical Service Laboratories, Toronto, Ontario).

Silicic acid impregnated papers were prepared according to the procedure of Lea, Rhodes, and Stoll (42). All chromatograms were developed with benzene: methanol (70:30; v/v) using ascending chromatography. The cholinec on-

taining lipids were detected by staining the chromatogram with the phosphomolybdic acid – stannous chloride reagent (43).

Egg lecithins were prepared essentially by the method of Hanahan (44). Lysolecithins were prepared by a combination of the methods of Hanahan (45) and Long and Penny (46). The fatty acid composition of the lysolecithins as determined by gas–liquid chromatography was: palmitic acid (72.1%), stearic acid (24.3%), and oleic acid (3.6%). Calculated for $C_{24.56}H_{53.04}NPO_8$ (521.3): C, 56.58; H, 10.26; N, 2.69; fatty acid, 50.6; P, 5.94; choline, 23.2. Found: C, 56.23; H, 10.12; N, 2.61; fatty acid, 50.4; P, 5.82; choline, 22.15; fatty acid:P = 1:1.01.

Lysolecithin cadmium chloride complexes were prepared in the following manner: lysolecithins (1 g) were dissolved in 95% ethanol (70 ml) by warming to 40°. A saturated ethanolic solution of cadmium chloride (30 ml) was added and the insoluble lysolecithin cadmium chloride complexes were removed by centrifugation. Dry weight = 1.2 g. Calculated for $C_{24.56}H_{53.04}NPO_8.CdCl_2$ (704.5): C, 41.86; H, 7.59; N, 1.99. Found: C, 41.6; H, 7.46; N, 1.90.

Preparation of Lecithins in Absence of Pyridine

The lysolecithin cadmium chloride complexes (1 g) were added to 20 ml of ethanol-free anhydrous chloroform, 1 ml of myristoyl chloride was added, and the mixture was shaken at room temperature for 3 hours. Chloroform (44 ml) was added to the reaction mixture and shaking was continued for 2 hours. The reaction mixture was added to 150 ml of chloroform:methanol:water (5:4:1; v/v) containing 49 mg of sodium bicarbonate and enough water and methanol were added to adjust the composition of the mixture to chloroform:methanol:water (5:4:1; v/v). After 30 minutes, the mixture was passed through a column of mixed bed resins IR-45 (60 ml) and IRC-50 (30 ml) to remove cadmium chloride and fatty acids, and the resins were washed with 200 ml of the solvent mixture. The total eluate was evaporated to dryness under reduced pressure and the residue was extracted with 80 ml of chloroform, any insoluble material being removed by centrifugation. The chloroform supernate was evaporated to dryness, 30 ml of acetone was added, and the acetone-insoluble fraction was removed by centrifugation. The two fractions were then dried and weighed: the acetone-soluble fraction (700 mg) contained 9.25 mg of phosphorus; the acetone-insoluble fraction (852 mg) contained 32.5 mg of phosphorus. The two fractions were chromatographed on silicic acid impregnated paper. The results indicated that the acetone-insoluble fraction contained almost exclusively lecithins and lysolecithins, whereas the acetone-soluble portion contained, in addition to these compounds, a substance that took up a position on the chromatogram between lysolecithin and lecithin.

The acetone-insoluble fraction was dissolved in 20 ml of benzene:methanol (70:30; v/v) and applied to a silicic acid column (2.4 cm diameter, 82 cm length) containing 100 g of silicic acid (Mallinckrodt 100 mesh). The column was developed with benzene:methanol (70:30; v/v). Fractions (20 ± 1 ml)

were collected and every fifth fraction was analyzed for total phosphorus. The course of chromatography is shown in Fig. 1. Chromatography of samples

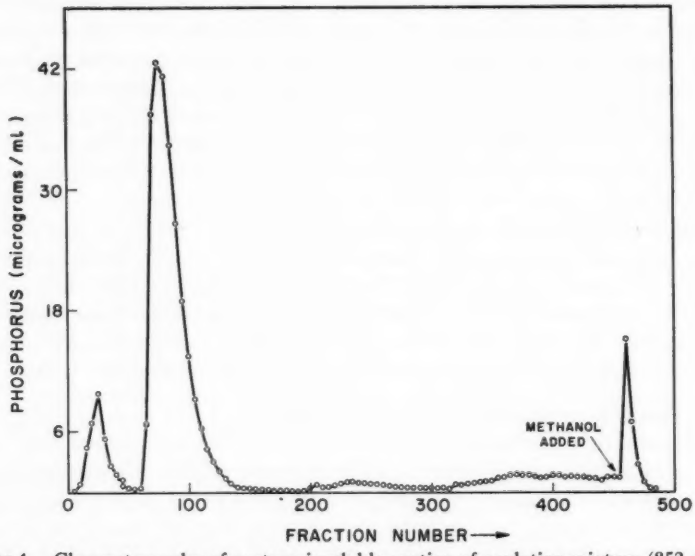

FIG. 1. Chromatography of acetone-insoluble portion of acylation mixture (852 mg ≡ 32.5 mg P) on a silicic acid column (100 g) with benzene: methanol (70:30).

from the four peaks on silicic acid impregnated paper indicated that peak 1 (fractions 5–50) had no choline-containing compounds, peak 2 (fractions 65–160) contained primarily lecithins (fractions 65–70 contained, in addition to lecithin, a substance chromatographically similar to lysolecithin), peak 3 (fractions 200–300) was a compound with chromatographic properties similar to sphingomyelin, and peak 4 (fractions 320–485) was lysolecithin. The fractions comprising peak 2 were combined into three groups, 65–75, 76–99, and 100–130, which weighed 135.4 mg, 290 mg, and 74 mg respectively. The material in fractions 76–99 (290 mg) contained myristic acid (49.8%), stearic acid (10.6%), and palmitic acid (39.6%). Calculated for $C_{38\cdot43}H_{78\cdot86}O_9NP$ (730): C, 63.3; H, 10.9; N, 1.92; fatty acids, 67.8; P, 4.24; choline, 16.6. Found: C, 63.55; H, 10.71; N, 1.93; fatty acid, 69.5; P, 4.21; choline, 16.35. $[\alpha]_D^{20} = +7.57 \pm 0.21$ (c, 11.1 in chloroform).

Action of Phospholipase A on "Semisynthetic" Lecithins Prepared in Absence of Pyridine

Lecithins (34.7 mg) from fractions 100–130 were dissolved in 110 ml of anhydrous diethyl ether containing 1.1 ml of 0.005 *M* calcium chloride. To this solution was added 4.7 mg of *Crotalus adamanteus* venom in 0.2 ml of 0.005 *M* calcium chloride followed by 10 ml of anhydrous ethyl ether. The

reaction was allowed to proceed for 16 hours, but no precipitate was present at this time. A further 4.5 mg of snake venom was added in 0.3 ml of 0.005 *M* calcium chloride solution together with a further 40 ml of ethyl ether; after 5 hours the flask contained a copious jellylike precipitate. The reaction was allowed to go for another 18 hours, 300 ml of ethyl ether was added, the flask was cooled at 5° C for 1 hour, and the lysolecithins were removed by centrifugation. The precipitate was dissolved in 300 ml of benzene:chloroform:methanol (3:1:2; v/v) and the enzyme removed by centrifugation. Samples of the lysolecithin fraction and the free fatty acid fraction were chromatographed on silicic acid impregnated paper. The chromatogram indicated that the reaction was 100% complete. Fatty acid analyses of the substrate and reaction products, carried out by gas–liquid chromatography, are given in Table I.

TABLE I

Fraction	Myristate (%)	Palmitate (%)	Stearate (%)
Original lecithins	48.95	46.0	5.05
Fatty acids freed	82.5	15.15	2.35
Lysolecithin fatty acids	14.8	75.6	9.6

Lecithins (35.15 mg) from fractions 76–99 were similarly degraded to lysolecithins and free fatty acids by phospholipase A. This reaction was also 100% complete.

Preparation of Lecithins in Presence of Pyridine

The procedure used in this experiment is similar to that described by Baer and Buchnea (3) for the acylation of L-α-glycerylphosphorylcholine cadmium chloride complex.

Lysolecithin cadmium chloride complexes (163 mg) were added to 5 ml of ethanol-free anhydrous chloroform followed by 0.8 ml pyridine (distilled from barium oxide) and 0.3 ml of myristoyl chloride. The reaction mixture was shaken at room temperature for 4½ hours and then 20 ml of ethanol-free chloroform was added. The solvent was removed under reduced pressure and the reaction mixture was placed in a vacuum desiccator overnight at a pressure of 0.1 mm. The reaction mixture was dissolved in chloroform:methanol:water (5:4:1; 30 ml) and was passed through a mixed bed resin (IR-45 (20 ml) and IRC-50 (10 ml)); the column was then washed with 70 ml of chloroform:methanol:water (5:4:1) and the total effluent was taken to dryness under nitrogen and reduced pressure, benzene:ethanol being added to facilitate removal of water. The reaction mixture was suspended in acetone (12 ml) and centrifuged; the precipitate was taken to dryness, suspended in ethyl ether (10 ml), centrifuged; and the precipitate was desiccated (weight = 75.8 mg).

The acylation product (75.8 mg) of lipid was dissolved in 10 ml of benzene:methanol (70:30) and was applied to a silicic acid (Mallinckrodt 100 mesh)

column (20 g) which was eluted with benzene:methanol (70:30). The course of chromatography was followed by phosphorus analysis. The material in the peak corresponding to the lecithins, when taken to dryness, weighed 23 mg.

A portion of the lecithins (6.9 mg) was hydrolyzed and the total fatty acids were analyzed by gas–liquid chromatography.

Action of Phospholipase A on Synthetic Lecithins Prepared in Presence of Pyridine

The remainder of the lecithins (16.1 mg) was dissolved in 100 ml of ethyl ether and subjected to phospholipase A action as described above. The reaction was quantitative as shown by chromatography on silicic acid impregnated paper. Fatty acid analyses of the substrate and reaction products are given in Table II.

TABLE II

Fraction	Myristate (%)	Palmitate (%)	Stearate (%)
Original lecithins	50.3	37.1	12.6
Fatty acids freed	88.9	8.2	2.9
Lysolecithin fatty acids	11.25	65.5	23.25

Discussion

The results of these studies indicate that lysolecithin cadmium chloride complexes can be acylated to the corresponding L-α-lecithins by the action of myristoyl chloride in a medium of chloroform, in the presence or absence of pyridine. The acylation in chloroform in the presence of pyridine has previously been reported by Hanahan, Brockerhoff, and Barron (8), and also de Haas *et al.* (12). However, the authors believe that this is the first time that lysolecithin cadmium chloride complexes have been acylated in the absence of pyridine. It is of interest to note that both these lecithins can be converted quantitatively to a mixture of lysolecithin and free fatty acids by the action of phospholipase A of snake venoms. This implies, since it is fairly well established that phospholipase A does not react with D-α-lecithins or β-lecithins (19), that no racemization or inversion occurs during the acylation of the lysolecithin cadmium chloride complexes. This fact tends to substantiate the conclusions of Baer and Buchnea (3) concerning racemization in the preparation of L-α-GPC by the methods of Tattrie and McArthur (1, 2) and Dawson (4).

Chromatography on silicic acid with benzene:methanol (70:30) leads to a partial fractionation of the lecithins as indicated by the fatty acid composition of the fractions from the center and end portions of the lecithin peak. The lecithins in the center portion of the peak (76–99) contain a higher percentage of stearic acid than those in the end portion (100–130). A similar observation that lecithins could be partially fractionated by means of silicic acid chromatography was made by Rhodes and Lea (47) when they reported that the faster-

running lecithins had a higher iodine value than the slower-moving lecithins.

Regarding the composition of the free fatty acids released from the semi-synthetic mixed lecithins by the action of phospholipase A, it may be seen from Tables I and II that more myristic acid is released in the case of the lecithin prepared in the absence of pyridine. If one assumes that the phospholipase A reacts specifically with the lecithin at a specific site, namely the acyl group attached to the β-carbon atom, then these observations may be explained on the basis of some acyl migration occurring during preparation of the lecithin in the presence of pyridine, and more acyl migration occurring during preparation of the lecithin in the absence of pyridine. However, if it is assumed that no acyl migration occurs during acylation in the presence of pyridine, and this is the commonly accepted view, then it is necessary to conclude that phospholipase A is specific in that it removes only one of the two acyl groups from lecithin but that this group may, to some extent, be removed from the α′- as well as from the β-position. This is essentially the interpretation that Marinetti *et al.* (34) placed upon their results. In the simplest interpretation the differences between the results with lecithins synthesized in the presence and absence of pyridine then indicate the extent of acyl migration that occurred in the absence of pyridine.

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DISTRIBUTION IN NATURE OF HEPTULOSE-PHOSPHATE-FORMING SYSTEMS[1]

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Abstract

The natural distribution was investigated of enzyme systems capable of forming heptulose phosphate from nucleosides, certain hexose phosphates, and ribose-5-phosphate. These systems occur widely in amphibia, birds, reptiles, and mammals. The materials examined included fungi (*Aspergillus niger*), bacteria (*Serratia mascerans*), yeast, germinating mung beans, spinach, and the brain, heart, kidney, liver, lung, intestine, egg, of fish, frog, turtle, chicken, rat, rabbit, mouse, and dog. Among the plants and microorganisms, activity towards nucleosides was absent (except for yeast) while that towards R5P and also G1P, G6P, and M6P was present.

Introduction

Three heptulose-forming systems can now be defined. In one, R5P[4] is converted to sedoheptulose phosphate by the well-known transketolase (1) enzyme; in the second, nucleosides are transformed into sedoheptulose phosphate (2); and in the third, G6P (as well as M6P, G1P) yields a new heptulose, 3-ketoarabo-heptose phosphate. Optimum conditions for each have been established (3, 4).

A favorable circumstance is the fact that the ketoheptoses and their phosphates all have the same molar extinction coefficient and the same absorption maxima in the Dische color reactions. Accordingly, it is convenient to determine the concentration of the three heptulose-phosphate-forming systems merely by measuring ketoheptose color in incubated digests containing the appropriate substrate.

Two important questions deserve attention. One is "How do these three systems compare in potency in the same biological source?" and the other is "What is the distribution in nature of heptulose-phosphate-forming systems?" Accordingly, a variety of forms of life, e.g., bacteria, fungi, yeast, plants, and a number of species of invertebrates and vertebrates, have been examined with a view to providing the desired factual answers. In the present paper, these experiments are described.

[1]Manuscript received March 23, 1961.

Contribution from the Tufts University School of Medicine and the New England Center Hospital, Boston, Mass. This work was aided in part by grants (P-106, P-107) from the American Cancer Society, Inc., New York, New York; (C-3213) from the National Cancer Institute, National Institutes of Health, Bethesda, Md. Presented at the 137th Meeting of the American Chemical Society, Inc., Cleveland, Ohio, April 9, 1960.

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[4]The following abbreviations are employed: R5P, ribose-5-phosphate; S7P, sedoheptulose-7-phosphate; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; M6P, mannose-6-phosphate; G1P, glucose-1-phosphate; 3-KAHP, 3-ketoarabo-heptose phosphate. A, adenosine; G, guanosine; I, inosine; U, uridine.

Experimental

Materials

Serratia mascerans 274, *Aspergillus niger*, and *Penicillum chrysogenum* were obtained from American Type Culture Collection, Washington, D.C. Bottom brewer yeast was kindly supplied by Haffenreffer Brewing Co., Boston, Mass.; the yeast was washed and dried at room temperature and kept in the cold room. *S. mascerans* was grown for 7 days on infusion broth of Baltimore Biochem. Lab. (25 g/liter). *A. niger* and *P. chrysogenum* were grown on potato-dextrose medium for 4 days, and the mycelia separated from the medium and used. Mung beans (*Phaseolus radiatus*) and spinach were obtained fresh locally. Bean sprouts were prepared by germination of the mung bean seeds in the laboratory for 48 hours, under a thin layer of water. Fish (*Tautoga Onitis*) was obtained fresh from a local grocer, and frog (*Rana pipiens*) and turtle from the animal farm of the Medical School. Live leghorn chickens were supplied by a poultry store. Albino rats and dog were the other animals used. Ehrlich mouse ascites tumor and Novikoff hepatoma were the source of neoplastic tissue for the study of heptulose phosphate synthesis. G1P, G6P, M6P, R5P, adenosine, guanosine, uridine, and inosine were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

Preparation of Heptulose-Phosphate-Forming Tissue Extracts

Aqueous homogenates of frog, fish, turtle, and other animal tissues in general were prepared (10% weight to volume) and centrifuged for 30 minutes at 18,000×*g*. The supernatants were used as an enzyme source. Mung bean sprouts (10 g) were homogenized with 5 ml of H_2O and centrifuged. The supernatant solution was employed. Spinach (70 g) was macerated with 30 ml water, centrifuged, and filtered. The filtrate contained the enzyme systems.

A. niger and *P. chrysogenum* mycelia were homogenized (1 to 10) with water, centrifuged, and filtered. *S. mascerans* (2 liters) was grown on nutrient medium for 7 days, harvested by centrifugation, macerated with sand, and the homogenate was centrifuged.

Systems for Measuring Heptulose Phosphate Formation

System 1: This was composed of 0.5 ml of 0.035 *M* nucleoside solution, 0.25 ml of enzyme solution, and 0.5 ml of phosphate buffer (0.01 *M*), pH 6.7.

System 2: This was composed of 0.2 ml of sugar phosphate (0.1 *M*), 0.5 ml of 0.05 *M* tris–maleate buffer, pH 7.2, and 0.3 ml of enzyme solution. The tubes were incubated at 37° for 4 hours under toluene, and then 1.25 ml and 1 ml of 95% ethanol were added for systems 1 and 2, respectively, and the mixture centrifuged. All analyses were performed on the supernatant.

Heptulose phosphate was determined on a 1.0-ml aliquot by the Dische orcinol reaction (5), using sedoheptulosan as a reference standard. In the experiments with mung bean enzyme, Dische's cysteine–H_2SO_4 (6) method was used for measuring heptulose phosphate.

Control digests were of two types. One lacked the enzyme and the other

lacked the substrate, and in both cases, buffer solution replaced the missing enzyme and substrate additions. No heptulose-reacting material was ever obtained in these control digests.

Results and Discussion

Heptulose Phosphate Formation from Nucleoside and Sugar Phosphate by Bacteria, Fungus, Yeast, Plant Enzymes

It was observed that *S. mascerans* 274, fungus *A. niger* and *P. chrysogenum*, mung bean seeds, 48 hours germinated seedling, and spinach do not contain the enzyme system which converts nucleoside to S7P, whereas yeast shows a very slight activity with guanosine and inosine. With sugar phosphate as substrate, *S. mascerans* has enzyme activity restricted to the formation of S7P from R5P. *A. niger* is completely inactive. It is interesting to note that bottom yeast has all the enzyme systems. In the plant kingdom, the S7P-forming system is absent in mung bean seed, but the systems requiring hexose phosphate as substrate do appear during the 48 hours of germination. It can be inferred that the enzyme is elaborated during the germination (7).

TABLE I

Survey of several microorganisms and plants for heptulose-phosphate-forming activity*

	Substrates							
	Nucleosides				Sugar phosphates			
	A	G	I	U	G1P	G6P	M6P	R5P
Serratia mascerans 274	0.0	0.0	0.0	0.0	0.0	0.0	0.0	426.0
A. niger	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Penicillinum chrysogenum	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1006.0
Bottom Brewer yeast	0.0	48.3	97.3	0.0	247.0	271.0	206.4	164.0
Mung bean seeds	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Mung bean sprout (48 hours)	0.0	0.0	0.0	0.0	90.7	107.8	62.5	125.0
Spinach	0.0	0.0	0.0	0.0	144.5	235.0	0.0	350.0

*The values are expressed as micromoles of heptulose formed per gram of tissue dry weight.

Spinach (goosefoot variety) lacks the enzyme synthesizing heptulose phosphate from M6P but not from G1P, G6P, or R5P. Horecker *et al.* (1) have purified transketolase from this source.

Heptulose Phosphate Formation by Tissue Enzymes of Fish, Frog, Turtle, and Chicken

Table II describes the activity of a number of tissues obtained from each species with regard to their ability to form heptulose phosphate from nucleoside and sugar phosphate. The common edible fish, tautog (*Tautoga onitis*), of wrasse family and commonly found in the North Atlantic waters, is used as representative for this species; the frog (*Rana pipiens*) is a member of the amphibians; a small sea turtle found in North Atlantic waters represents a member of the reptile family; and a Leghorn chicken, an avian form of life.

TABLE II

Heptulose phosphate formation by tissues of fish, frog, turtle, and chicken*

	Substrates							
	Nucleosides				Sugar phosphates			
	A	G	I	U	G1P	G6P	M6P	R5P
Fish								
Brain	127.0	209.0	212.0	0	122.5	123.5	123.5	608.0
Heart	0.0	0.0	0.0	0	0	0	0	0
Kidney	558.0	828.0	593.0	0	703.0	483.0	512.0	625.0
Liver	212.0	610.0	337.0	0	598.0	428.0	368.0	356.0
Spleen	508.0	300.0	278.0	0	558.0	493.0	513.0	397.0
Intestine	390.0	616.0	432.0	0	244.0	264.0	181.5	934.0
Frog								
Brain	156.0	98.3	320.0	71.3	924.0	1174.0	1262.0	1274.0
Heart	0	0	0	0	101.5	420.0	264.0	643.0
Kidney	672.0	280.0	733.0	159.0	955.0	1100.0	843.0	505.0
Liver	13.3	28.7	85.6	0	350.0	507.0	469.0	286.0
Lung	111.9	0	64.5	0	427.5	787.5	486.5	973.0
Intestine	96.0	27.0	55.4	0	203.3	183.1	188.7	222.9
Egg	0	12.2	29.9	0	350.7	476.0	254.7	789.0
Turtle								
Brain	81.0	270.0	216.0	172.0	1100.0	1002.6	1542.0	1920.0
Heart	0	0	0	0	110.0	147.5	135.0	386.0
Kidney	1165.0	403.0	675.0	0	1642.0	1480.0	1369.0	1359.0
Liver	430.0	615.0	548.0	0	793.0	673.0	528.0	958.0
Lung	190.5	214.2	258.0	0	160.5	193.0	128.5	607.0
Intestine	1305.5	1560.0	1500.0	0	1220.0	2390.0	1395.0	3515.0
Egg	2410.0	1435.0	2020.0	0	2090.0	2390.0	2050.0	4625.0
Chicken								
Brain	321.0	296.0	391.0	370.0	1022.0	973.0	1092.0	964.0
Heart	0	0	0	0	5.9	0	0	71.7
Kidney	114.0	101.6	138.0	0	415.0	504.0	466.0	504.0
Liver	122.0	169.4	124.3	47.5	73.5	88.8	62.3	241.8
Lung	55.2	55.2	127.0	0	279.9	435.0	232.0	660.0
Spleen	242.0	151.5	160.0	113.2	523.0	662.0	608.0	394.0
Intestine	314.0	408.0	365.0	110.0	890.0	1380.0	1290.0	2040.0
Egg	0	0	0	0	0	0	0	0

*The values are expressed as micromoles of heptulose formed per gram of tissue dry weight.

The different animal species showed similar enzyme activity with regard to heptulose phosphate synthesis with only minor variations. Thus, in each species, kidney, intestine, and spleen were the organs with most concentrated enzyme activity. Heart was uniformly deficient in this activity. Among the nucleosides, uridine was the poorest substrate, while purine nucleoside showed a much greater transformation to heptulose phosphate, to a degree which varied depending on the tissue.

The only point of major difference between these species is the presence of a rather high enzyme activity towards nucleosides in turtle eggs, and its absence in frog and chicken eggs. Chicken eggs also showed the absence of activity with sugar phosphate.

Heptulose Formation by Mammalian Species

Mice, rat, rabbit, and dog were chosen for this study. These four laboratory

TABLE III

Ability of tissues of mice of two inbred strains to produce heptulose phosphate*

	Substrates							
	Nucleosides				Sugar phosphates			
	A	G	I	U	G1P	G6P	M6P	R5P
Ajax								
Brain	203.0	340.0	506.0	0.0	832.0	1044.0	832.0	544.0
Heart	271.0	219.0	583.0	0.0	934.0	990.0	753.0	728.0
Kidney	548.0	472.0	780.0	0.0	1053.0	913.0	493.0	1013.0
Liver	452.0	563.0	612.0	182.0	526.0	603.0	334.0	468.0
Lung	1190.0	427.0	525.0	0.0	825.0	917.0	705.0	903.0
Spleen	1592.0	303.0	763.0	0.0	892.0	1060.0	827.0	472.0
Intestine	1248.0	558.0	513.0	147.7	780.0	790.0	782.0	424.0
C3H								
Brain	196.0	475.0	623.0	63.5	1300.0	1490.0	1300.0	1430.0
Heart	0.0	250.0	792.0	76.3	596.0	475.0	698.0	543.0
Kidney	715.0	503.0	385.0	0.0	1038.0	973.0	949.0	452.0
Liver	483.0	614.0	711.0	90.0	787.0	563.0	595.0	306.0
Lung	1132.0	278.0	1100.0	106.0	873.0	970.0	827.0	1151.0
Spleen	2000.0	0.0	1195.0	116.8	1296.0	1143.0	980.6	503.3
Intestine	893.0	670.0	613.0	0.0	518.0	627.0	536.0	317.0

*The values are expressed as micromoles of heptulose formed per gram of tissue dry weight.

TABLE IV

Heptulose phosphate formation by tissues of the rat, rabbit, and dog*

	Substrates							
	Nucleosides				Sugar phosphates			
	A	G	I	U	G1P	G6P	M6P	R5P
Rat								
Hemolysate					20.3	55.5	27.2	126.0
Brain	166.5	557.0	528.0	55.9	534.0	660.0	735.0	1825.0
Heart					265.0	292.0	269.0	343.0
Kidney	1110.0	670.0	413.0	762.0	730.0	538.0	565.0	480.0
Liver	399.0	805.0	542.0	109.2	514.0	360.0	344.0	209.0
Lung	1155.0	482.0	438.0	183.5	726.0	620.0	608.0	662.0
Spleen	1800.0	640.0	424.0	152.5	408.0	423.0	450.0	361.0
Intestine	816.0	552.0	362.0	177.0	464.0	324.0	404.0	436.0
Rabbit								
Hemolysate	20.6	9.1	4.85	3.24	29.4	52.0	24.3	31.4
Brain	785.0	936.0	892.0	0.0	525.0	1305.0	1040.0	2540.0
Heart	1.0	0.0	0.0	0.0	27.8	194.0	139.0	246.0
Kidney	174.5	271.0	276.0	152.5	903.0	957.0	752.0	585.0
Liver	130.5	444.0	448.0	85.7	512.0	571.0	441.0	431.0
Lung	337.0	303.0	409.0	29.4	703.0	743.0	499.0	1185.0
Spleen	125.0	393.0	394.0	0.0	758.0	868.0	572.0	317.0
Intestine	291.0	983.0	1002.0	253.0	568.0	247.0	512.0	438.0
Dog								
Hemolysate	0.0	0.0	0.0	0.0	60.4	74.5	41.3	36.8
Brain	130.0	1200.0	930.0	0.0	410.0	1482.0	816.0	2600.0
Heart	201.0	327.0	475.0	0.0	735.0	770.0	770.0	1123.0
Kidney	1296.0	282.0	410.0	0.0	1965.0	1462.0	1272.0	672.0
Liver	1684.0	1002.0	775.0	225.0	1955.0	1546.0	1378.0	915.0
Lung	137.0	408.0	418.0	0.0	1098.0	1935.0	1028.0	777.0
Spleen	375.0	1075.0	712.0	190.5	2670.0	2710.0	2220.0	2350.0
Intestine	362.0	1715.0	1182.0	0.0	1052.0	1206.0	798.0	736.0

*The values are expressed as micromoles of heptulose formed per gram of tissue dry weight.

animals showed the presence of enzymes for the synthesis in most of their tissues (Tables III and IV). The similarity between the four animals is evident and no qualitative differences can be seen. There are, however, some variations in the amount of heptulose synthesized by preparations from the different species. Thus, with regard to the nucleosides, spleen of mouse, rat, and dog liver are highly active with adenosine as substrates. With regard to the sugar phosphates, dog spleen exhibited the strongest activity toward all four substrates.

TABLE V

Ability of two transplantable tumors to produce heptulose phosphate*

	Substrates							
	Nucleosides				Sugar phosphates			
	A	G	I	U	G1P	G6P	M6P	R5P
Mouse Erhlich ascites tumor	27.6	15.2	51.5	0.0	806.0	772.0	622.0	712.0
Novikoff hepatoma	202.7	166.7	152.0	0.0	285.0	346.0	132.0	180.0

*The values are expressed as micromoles of heptulose formed per gram of tissue dry weight.

Heptulose Formation by Neoplastic Tissues

Seven-day-old Ehrlich mouse ascites tumor cell and 7-day-old Novikoff hepatoma were tested for their heptulose-phosphate-synthesizing capacity. It was found that nucleoside activity is low in Ehrlich ascites cells, whereas sugar phosphate activity corresponds to that of normal tissues. In Novikoff hepatoma, activity for nucleoside and sugar phosphate was lower than in normal rat liver. The data are presented in Table IV. Colajacomo *et al.* (8) also observed nonoxidative heptulose formation from sarcoma 181 ascites and Ehrlich's solid carcinoma from G6P.

In conclusion the data indicate the presence of heptulose-phosphate-producing systems in many forms of life, from primitive unicellular organisms to highly developed mammalian species. This widespread distribution of all three heptulose-phosphate-forming systems suggests a significant biological role for these enzymes.

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THE ESTIMATION OF FIBROUS CONNECTIVE TISSUE IN LIVER BY A CHEMICAL METHOD[1]

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Abstract

The chemical determination of hydroxyproline has been studied and a modification of the method of Neuman and Logan has been applied to hepatic tissue. The content of hydroxyproline in the livers of rats that had been fed experimental diets has been found to correlate well with the amounts of fibrous connective tissue (structural reticulin plus newly formed collagen) as estimated histologically.

Introduction

In experimental studies involving the effect of diet or drugs on the liver, an accurate, objective method for determining the degree of the fibrosis or cirrhosis would be valuable. Conventional methods involve selective staining of the structural reticulin fibers and newly formed collagen followed by a subjective assessment of the degree of histological abnormality. It is well known to pathologists that sections from different parts of the same liver may show markedly different amounts of fibrosis, or of other change. Even when regional differences are not large, the subjective impressions of different observers sometimes lead to rather divergent reports as to the amount of fat, fibrous connective tissue or other abnormality present.

Earlier attempts to estimate the amount of fibrous connective tissue in the organs of animals depended upon the insolubility of collagen and elastin, relative to other tissue proteins and cellular components, in dilute solutions of sodium hydroxide (0.1 *N*) at room temperature (1–9). Such procedures suffer from the disadvantage that several types of collagen and other connective tissue material appear to exist, with different tendencies to be attacked by cold dilute alkali (cf. 6, 9).

The uniquely high content of hydroxyproline in connective tissue was commented upon by Neuman and Logan (10, 11) and used by them as a more specific means of estimating the amount of collagen in various tissues. The present studies sought to establish whether a chemical determination of hydroxyproline in hepatic tissue would indeed provide a method that is free from bias for estimating the average degree of cirrhosis or at least of total fibrous connective tissue in a sample of liver. The procedure studied and modified was that of Wierbicki and Deatherage (12). This method depends upon the oxidation of hydroxyproline with alkaline hydrogen peroxide to yield

[1]Manuscript received April 7, 1961.
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a pyrrole which forms an intensely red color with *p*-dimethylaminobenzaldehyde (PDMAB). Neuman and Logan (11) suggested the use of aqueous urea for the preliminary extraction of non-fibrous proteins from tissues. Although this technique reduced the amount of tyrosine in the digests, it offered no great advantage in the experience of Wierbicki and Deatherage or of ourselves. The values for the hydroxyproline content of normal liver tissue before and after extraction with urea were found to be virtually identical (2.23 ± 0.04 vs. $2.18 \pm 0.04\%$). The amounts of hydroxyproline found (by K.K.G.M.) in about two hundred livers showed a good correlation with estimates of fibrosis made by histological methods (by W.G.B.C.). After the work was completed (in 1956) the two authors mentioned left Toronto and there has been a long delay in preparing the data for publication.

Meanwhile, Miyada and Tappel (13) suggested modifications that eliminated some of the difficulties mentioned by Baker, Lampitt, and Brown (14). Fels (15) has described what appear to be several improvements in the determination of hydroxyproline in liver but these have not been tested here. More recently, Hutterer and Singer (16) have suggested other minor changes said to stabilize the color.

Experimental

Preliminary Procedures

The sampling problem, which has already been mentioned as a source of uncertainty in the conventional histological method of estimating liver damage, provides the major source of variability in the analytical results when the chemical method is used to estimate hepatic fibrosis. If the whole liver could be hydrolyzed, a true mean value for hydroxyproline could be determined. For livers of larger animals such a procedure would be both expensive and laborious. Even with smaller animals it is often not feasible since some hepatic tissue is usually required for histological examination. In the case of a 200-g rat, for example, whose liver normally weighs about 8–10 g, our usual procedure involves removal of several portions, amounting to 0.8–1.5 g, for miscroscopic study. The remainder (85–90% of the total) is extracted with acetone and hot alcohol to determine hepatic lipids and the dry, fat-free tissue residue (DFFR, weighing 1.3 to 2.2 g) is available for the determination of hydroxyproline. It is almost impossible to grind manually this fibrous residue to a fine powder, not only because of its leathery character but because any fines that are produced acquire a charge and are difficult to keep from flying away. A high-speed cutting–grinding machine* which reduced all the liver residue to a very fine powder (through a 200-mesh sieve) was made in our shop.

To 100–120 mg of the finely ground DFFR in a pyrex test tube, were added successively 1 ml water to wash down any particles adhering to the sides of the tube, 2 ml of 10 *N* hydrochloric acid, and 0.2 ml (containing 50 mg) of

*The machine resembles in many respects the micromodification of a Wiley mill described by W. H. Cook, E. P. Griffing, and C. L. Alsberg, (Ind. Eng. Chem. Anal. Ed. **3**, 102 (1931)).

stannous chloride. The tube was sealed and heated for 6 hours at 50-lb gauge pressure (148° C) in a steam autoclave. The acid digest was neutralized with 2.5 *N* sodium hydroxide, filtered, and the filtrate was made to a suitable volume, usually 50 ml. The hydroxyproline in 1.00-ml aliquots of the neutralized digest was oxidized with alkaline hydrogen peroxide to yield a pyrrole which then forms an intensely red color with *p*-dimethylaminobenzaldehyde (PDMAB).

Summary of the Modified Colorimetric Procedure

To 1.00 ml of neutralized acid digest of tissue (corresponding to about 2 to 5 mg DFFR), add 1.0 ml of 0.06 *M* solution of copper sulphate, 1.0 ml of 2 *N* sodium hydroxide, and 1.0 ml 6% hydrogen peroxide, in that order. Heat the tubes at 80° C for 10 minutes and cool in ice. To the clear solution add 4.0 ml 2.5 *N* sulphuric acid and 2.0 ml 2% solution of PDMAB in *n*-propanol. After mixing well, heat the tubes in a water bath at 70° C for exactly 13 minutes. Cool the reaction mixture immediately (cold water) and read the optical density at 555 mμ.

Standard Graph

A straight line was obtained when optical density was plotted against hydroxyproline in the range 2 to 30 μg.

Corrections for Trytophane and Tyrosine

Tryptophane and tyrosine also develop color with PDMAB under these conditions. On a weight basis they give rise to 0.6% and 1.2% as much color, respectively, as does hydroxyproline. A correction for tryptophane is, however, unnecessary in acid hydrolyzates. Tyrosine in the hydrolyzates was determined by the method of Folin and Ciocalteau (17) and the values for hydroxyproline were estimated after subtracting the color due to tyrosine.

Histological Evaluation of Cirrhosis (Fibrosis)

The histological grading of cirrhosis (fibrosis) was done on sections stained by Mallory's trichrome method. A progressive increase of connective tissue accompanied the changes described below.

Cirrhosis +: Accentuation of the hepatic pattern by interlacing bands of connective tissue in the major part of the section.

Cirrhosis ++: A definite disruption of the lobular pattern with a tendency to nodule formation.

Cirrhosis +++: Well-established nodularity diffusely present throughout the section.

Cirrhosis ++++: The section is either (1) broken up into numerous small nodular areas or else (2) broken up into large and small nodules with replacement of considerable areas of parenchyma by connective tissue.

Results and Discussion

Two technical changes in the determination of hydroxyproline in liver, proposed by Fels (15) after our work was completed, appear to be improve-

ments and should be mentioned although we have no experience with them. Fels eliminates the interference caused by the larger amounts of tyrosine in parenchymous tissues than in collagenous material, by preferential adsorption of tyrosine on a specially prepared Norit. He has simplified the mechanics of the hydrolytic procedure by using serum vials sealed with rubber and aluminum caps applied with a hand crimper. Hutterer and Singer (16) increased the concentration of *n*-propanol in the reaction mixture and claimed that improved stability of color resulted.

A summary of the histological evaluations of connective tissue and of the chemically determined hydroxyproline values from seven nutritional experiments is presented in Table I. The data in the first five columns were obtained from the livers of rats that had been fed diets poor in choline and (or) protein for periods of from 2 to 12 months. The last two sets of data are from rabbit livers. The few values shown for normal rat livers are about twice those (1.26 ± 0.13 mg/g DFFR) reported by Fels (15). Differences in strain of rat or in diet may account for this.

A positive correlation is obvious in Table I between the histologist's estimates of the extent of connective tissue change and the mean values for the amount of hydroxyproline found by chemical analysis in the livers grouped

TABLE I

Comparison between histological evaluation of connective tissue changes and hepatic hydroxyproline
(Figures in parentheses represent the number of livers examined)

	Average amount of hydroxyproline found (mg/g DFFR)						
Expt.	YGA	YGB	NTL	NTM	NTP	TR	TRA
Cirrhosis (fibrosis) reported							
0		2.45 (28)			3.35 (5)	1.85 (3)	2.03 (4)
±	1.91 (2)	2.93 (5)		3.25 (3)	3.66 (2)		
+		2.32 (5)	4.09 (4)	4.32 (4)		4.40 (2)	4.58 (7)
+±		3.88 (2)	4.50 (14)	4.89 (5)	4.82 (3)		
++	3.46 (5)	4.32 (4)	4.51 (7)	4.43 (4)		6.77 (4)	5.00 (3)
++±		4.39 (3)	5.36 (10)	4.72 (5)			
+++	3.74 (3)		5.97 (10)		5.64 (3)	5.47 (1)	8.40 (2)
+++±	4.40 (9)	6.35 (4)	6.62 (6)		6.39 (1)		
++++	4.86 (5)	6.54 (3)	7.75 (3)		6.81 (1)	9.26 (2)	

according to the histological reports. Figures 1 and 2 show a considerable range of values for hydroxyproline in livers possessing similar amounts of fibrous connective tissue by visual estimation. The range of variation and consequent imperfect correlation is probably due mainly to sampling errors rather than to errors of judgment on the part of the microscopist. It is unreasonable to assume that one, or even several, specimens of hepatic tissue a few square centimeters in area and a few microns thick necessarily provide a typical sample of the liver. Because in this study the chemical assay for

hydroxyproline was performed on a representative sample (viz. the major portion of the hepatic tissue) it undoubtedly gives a more reliable estimate of the over-all degree of fibrosis than one based on examination of a few sections. A careful histological study of an equal bulk of the liver tissue would doubtless give as good an assessment of the hepatic damage, but it would involve a great deal more time and expense. It is a truism (not always remembered) that *any* method that purports to indicate the extent of pathological change in an organ must have, as a prime requisite, enough material to constitute a reasonably representative sample. The chemical procedure appears able to reduce the amount of work involved in making an adequate assessment. More study will be required before one learns fully how to correlate the time-honored histological assessment of connective tissue changes with the chemical findings.

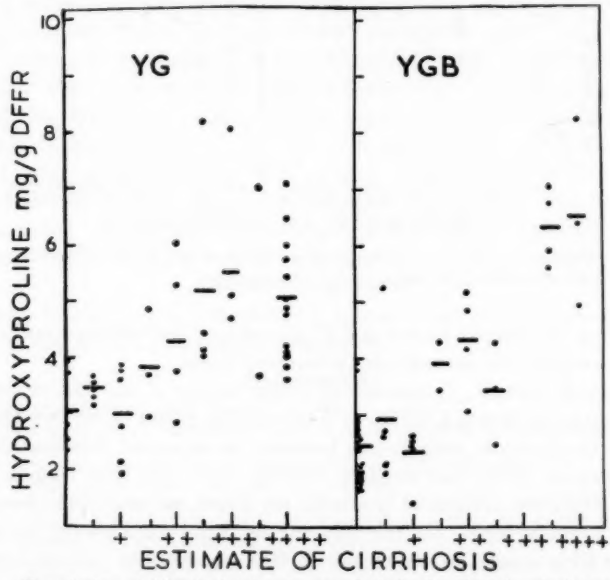

FIG. 1. Correlation of histologists' estimate of cirrhosis with chemical determination of the amount of hydroxyproline present in the dry, fat-free hepatic tissue residue of rats fed a hypolipotropic diet.

All connective tissues do not possess the same ratio of collagen to elastin. Quantitative data on this are limited. Elastin contains 1.5 to 2.3% hydroxyproline, depending on the source (10, 11); collagen and gelatin (its partial hydrolysis product) contain $14.42 \pm 0.25\%$ (12). Thus the factor for elastin would be "hydroxyproline found $\times (100/1.9) = 53$", and for collagen it would be $(100/14.42) = 6.94$. Wierbicki and Deatherage prepared five different samples of connective tissue (alkali-insoluble proteins) from meat. They found the hydroxyproline content of beef muscle connective tissue to be $12.39 \pm 0.40\%$

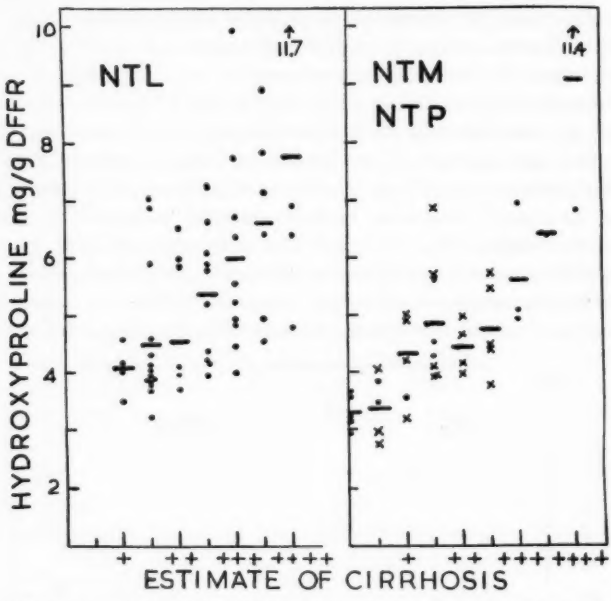

FIG. 2. Similar correlation of histological estimates of degree of cirrhosis with chemical determinations of hydroxyproline.

and therefore proposed a factor of 8.07 to convert the hydroxyproline found in meat to the equivalent amount of connective tissue.

The livers of normal (?) rats of our strain appear to contain on the average 2.6 mg hydroxyproline/g DFFR. If one uses the factor 8 for hepatic connective tissue, until a more reliable one has been determined, this would represent about 21 mg of connective tissue/g DFFR. The DFFR is usually about 20 to 24% of the fresh weight of (normal) rat livers, so that if the factor 8 does apply, an average figure for the amount of connective tissue/100 g of fresh normal rat liver would be about 400 to 500 mg. Similar calculations may be made for livers with fatty and fibrotic changes. The lowest value found for hydroxyproline in any liver graded as ++++ fibrosis was 3.6 mg/g in one rat of experiment YG. The lowest value associated with ++++ grading in experiment NTL was 4.6, and in experiment YGB it was 4.95 mg/g. Values for hydroxyproline above about 6.5 mg/g DFFR appear, from Table I, to be characteristic of livers rated at fibrosis ++++, i.e. 50 mg of connective tissue/g DFFR would seem to represent an amount of fibrotic change beyond which it is difficult to distinguish differences in degree by visual inspection. It may be interesting to note that this amount of connective tissue is barely three times the normal amount, if one assumes the same ratio of elastin to reticulin to collagen in the initial and final states.

Until more is known about the histochemistry and histopathology of the changes that occur in hepatic connective tissue, the use of factors should be avoided. Meanwhile an adequate comparative rating of the amount of connective tissue present in any sample of liver would appear to be best expressed as milligrams of hydroxyproline/gram DFFR, since these data show a good correlation with the findings upon histological examination.

Aside from the difficulties arising out of the differing hydroxyproline contents of different types of connective tissue (elastin, reticulin, and collagen) and the fact that the proportions of these may vary as the hepatic lesions become more severe, the basic problem remains of how to express the results. We have discussed this previously (18, 19). Because large changes may occur in the amounts of fat or of glycogen present in the liver, as well as in the free and bound water, hydroxyproline contents reported as percentage of fresh liver weight or even of DFFR may not give a realistic picture of the relative amounts of connective tissue present. Kosterlitz and Campbell (20) have proposed that the amounts of variable components of liver tissue be expressed in terms of the DNA content since this is one component of the protoplasmic system that seems to maintain a constant relationship to the number of metabolically active cells in the liver. Data on the DNA content of these samples of DFFR are being determined and the findings will be reported shortly, as will a more detailed report of the histological findings mentioned above.

Acknowledgments

This work was supported in part by grants from the National Research Council and the Banting Research Foundation.

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EFFECTS OF INCUBATION CONDITIONS ON THE IN VITRO ASSAY METHOD FOR INSULIN[1]

D. C. JESSUP AND G. S. WIBERG

Abstract

A number of factors such as incubation time, glucose concentration, and pre-incubation cooling affect the deposition of glycogen and the uptake of glucose by diaphragm sections in response to insulin. The greatest response occurs during the first 30 minutes of incubation but for assay purposes an incubation period of 90 minutes was recommended as it gave the greatest difference between control and treated tissues. A glucose concentration of 0.2% in the medium was found optimum. The effect of insulin at higher and lower concentrations was not as great. Preincubation cooling had an adverse effect on the response of the tissues to insulin.

Introduction

In a recent publication, Jessup and Wiberg (1) compared two parameters of insulin activity to determine which offered the greater suitability as a response criterion. The responses investigated were (i) glucose uptake and (ii) glycogen deposition in an eighth section of isolated rat diaphragm. On the basis of studies carried out, glycogen deposition appeared to be the superior parameter for the measurement of insulin activity.

The variables studied in the initial investigation included tissue size, between-animal and within-animal variation, and the dose–response relationship. The conditions of incubation were held constant and reflected the generally accepted recommendations for maximum glucose uptake (2, 3). It has been shown (4, 5) that alteration of the incubating conditions affects the utilization of glucose but the effect on the glycogen deposition has received only limited consideration (6, 7). In the present report, the conditions of tissue size and insulin concentration were held constant while the conditions of incubation were varied in order to establish the optimal response for glycogen deposition, in the presence and absence of exogenous insulin.

Methods

The assay procedure described previously (1) measured glucose uptake and glycogen deposition by isolated sections of a single rat diaphragm. The diaphragm was divided into eight approximately equal parts and each section was incubated separately in 1.0 ml Krebs–Ringer bicarbonate (KRB) solution in the presence of glucose and insulin. In the present investigation, to establish the optimum condition, glucose concentrations were varied but a single dose level (0.5 milliunit per ml) of insulin was utilized throughout.

Glucose uptake was determined by measuring the glucose remaining in the

[1]Manuscript received April 28, 1961.
Contribution from the Physiology and Hormones Section, Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Canada.

medium after incubation, by the anthrone method (8). Glycogen deposition was determined by extracting the tissues with 5% trichloroacetic acid and measuring glycogen concentration with anthrone reagent (9). Results were expressed as milligrams of glucose or glycogen per gram of wet tissue.

Using these techniques, a series of experiments were carried out to determine (i) the rate of breakdown of glycogen in tissue when no glucose or insulin was present, (ii) the effect of time of incubation on glycogen deposition and glucose uptake, (iii) the effect of glucose concentration in the medium on the two response criteria, (iv) the effect of chilling the tissue prior to incubation, and (v) the glucose concentration permitting the greatest insulin effect.

Results

1. Glycogen Depletion during Incubation

Diaphragms were removed from seven rats, one each day for 7 days, and each diaphragm was sectioned into eight approximately equal portions. Each section of each diaphragm was incubated separately in Krebs–Ringer bicarbonate solution without glucose or insulin for a specific length of time.

Glycogen determinations were carried out after 0, 30, 60, and 90 minutes of incubation. These times were measured after the 10-minute gassing period with 95% O_2–5% CO_2.

Table I presents the results of these experiments which showed that glycogen concentration in the tissues was reduced as the incubation time increased. Greatest depletion occurred during the first half hour.

TABLE I

Glycogen depletion in tissues incubated without glucose or insulin for different periods of time

Incubation time, minutes	No. of determinations	Glycogen concentration, mg/g wet wt.	Glycogen depletion,† mg/g
Non-incubated	14	2.55±0.67*	—
30	14	1.56±0.42	0.99
60	14	1.14±0.28	1.41
90	14	0.99±0.38	1.56

*Standard error of the mean.
†Non-incubated minus incubated.

2. Time of Incubation

Isolated diaphragm sections were incubated in either KRB–glucose solution or in KRB–glucose–insulin solution. The glucose concentration of the medium was 2.0 mg per ml; the insulin concentration was 500 microunits per ml. Following incubation for time periods of 0 to 120 minutes after gassing, glucose and glycogen determinations were carried out on the media and tissues respectively.

The results of these assays are shown in Table II. Glucose uptake increased with time up to 120 minutes in the absence of insulin but, with insulin present,

TABLE II

Effect of time of incubation on glucose uptake and glycogen deposition

Incubation time in minutes	Glucose uptake, mg/g		Glycogen concn., mg/g	
	Insulin, 0.0 milliunit/ml	Insulin, 0.5 milliunit/ml	Insulin, 0.0 milliunit/ml	Insulin, 0.5 milliunit/ml
0	0.0	1.54 ± 0.29* (10)†	2.20 ± 0.22* (10)†	3.38 ± 0.22* (10)†
30	3.89 ± 1.03* (10)†	7.26 ± 0.95 (10)	3.06 ± 0.23 (10)	4.15 ± 0.48 (10)
60	4.10 ± 0.67 (10)	10.15 ± 1.20 (10)	3.27 ± 0.35 (10)	5.67 ± 0.42 (10)
90	5.63 ± 1.15 (10)	15.11 ± 1.06 (10)	3.12 ± 0.28 (10)	6.36 ± 0.75 (10)
120	8.60 ± 1.37 (8)	12.15 ± 0.84 (8)	2.33 ± 0.19 (8)	5.87 ± 0.56 (8)

*Standard error of the mean.
†Number of determinations.

maximum uptake occurred at 90 minutes. Glycogen concentration reached a plateau after 30 minutes of incubation without insulin. In the presence of insulin, glycogen concentration leveled off at 60 minutes.

3. Effect of Glucose Concentration on Glucose Uptake and Glycogen Deposition

Single rat diaphragms were sectioned on each of 5 days. Two sections of diaphragm were incubated separately with each of three concentrations of glucose in KRB solution; the remaining two sections were incubated each with a different concentration of glucose. The five concentrations of glucose in KRB solution were 0.5, 1.0, 2.0, 4.0, and 8.0 mg per ml. The tissues were all incubated for 90 minutes and eight determinations of glucose uptake and glycogen deposition were made at each concentration.

The results presented in Table III indicate that both glucose uptake and glycogen deposition increase with increasing glucose concentrations up to between 4.0 mg and 8.0 mg per ml where glycogen deposition levels off and glucose uptake approaches a maximum. In the absence of insulin a glucose concentration of 4.0 mg per ml appeared to be most effective for both response criteria.

TABLE III

Effect of glucose concentration on glucose uptake and glycogen deposition in the absence of insulin

Glucose concentration, mg/ml	No. of determinations	Glucose uptake, mg/g/90 minutes	Glycogen deposition, mg/g/90 minutes
0.5	8	1.96±0.24*	1.55±0.31*
1.0	8	4.07±0.46	1.90±0.27
2.0	8	6.96±1.21	2.57±0.41
4.0	8	12.95±1.88	3.48±0.55
8.0	8	15.06±2.13	3.43±0.27

*Standard error of the mean.

4. Effect of Insulin at Optimum Glucose Concentrations

To determine whether or not the glucose concentration of 2.0 mg/ml, employed in the assay procedure described by Jessup and Wiberg, 1961, was as effective as 4.0 mg/ml, another series of tests was performed.

The diaphragms were removed from four rats, one on each of 4 days. Each diaphragm was sectioned into eight portions and each section was incubated separately. Four sections were incubated separately in KRB solution containing 2.0 mg of glucose per ml while the other four portions were individually incubated with 4.0 mg of glucose per ml of medium. Further, in two flasks at each glucose concentration, insulin (0.5 milliunit per ml) was present.

Glucose uptake and glycogen deposition were determined in each case. The difference between control and insulin-treated tissue responses gave a measure of the insulin effect. These results are presented in Table IV where it can be seen that the greatest percentage increase attributable to insulin occurred in the medium containing 2.0 mg of glucose per ml.

TABLE IV

Effect of insulin on glucose uptake and glycogen deposition at optimum glucose concentrations

Incubating medium	Glucose uptake, mg/g/90 minutes	Excess uptake (insulin effect)	Glycogen deposition, mg/g/90 minutes	Excess deposition (insulin effect)
KRB + glucose (2.0 mg/ml)	3.66 ± 0.46*	—	2.38 ± 0.27	—
KRB + glucose (2.0 mg/ml) + insulin	9.19 ± 1.66	5.53 (151%)	5.46 ± 0.65	3.08 (130%)
KRB + glucose (4.0 mg/ml)	8.52 ± 1.60	—	3.09 ± 0.36	—
KRB + glucose (4.0 mg/ml) + insulin	13.92 ± 1.78	5.40 (63%)	6.06 ± 0.94	2.97 (98%)

*Standard error of the mean.

5. Effect of Preincubation Cooling

Rat diaphragms were again sectioned into eight portions. Two sections were immediately placed in KRB–glucose–insulin solution and incubated. The glucose concentration was 2.0 mg per ml and the insulin concentration was 0.5 milliunit per ml.

The second, third, and fourth pairs of tissue sections were immersed in ice-cold KRB solution, containing no glucose or insulin, for time intervals of 10, 20, and 30 minutes respectively. At the end of each cooling period two portions of tissue were removed, blotted, and placed in KRB–glucose–insulin media for incubation. After 90 minutes of incubation, glucose uptake and glycogen deposition were determined.

Table V contains the results of a number of assays done in this manner. It will be noted that both response criteria are greater when no preincubation

TABLE V

Effect of preincubation cooling on glucose uptake and glycogen deposition by tissues incubated with 0.5 milliunit of insulin

Cooling time in minutes	Glucose uptake, mg/g wet tissue	Glycogen concentration, mg/g wet tissue
0	10.05 ± 0.69* (10)†	4.70 ± 0.28* (10)†
10	8.68 ± 0.64 (10)	3.29 ± 0.30 (10)
20	7.39 ± 1.06 (8)	3.21 ± 0.44 (8)
30	8.02 ± 0.63 (10)	3.37 ± 0.44 (10)

*Standard error of the mean.
†Number of determinations.

cooling takes place. The response to insulin of cooled tissue is significantly less ($P < 0.01$) when glycogen deposition is measured; the response when glucose uptake is measured is also less ($0.05 > P > 0.02$).

Apparently the loss of activity occurs during the first 10 minutes of cooling.

Discussion

Variations in incubation conditions may affect the response of tissue to insulin. It has been seen that tissue glycogen becomes depleted significantly

when the tissue was incubated for 30 minutes or more in a physiological medium containing no glucose. When glucose was present, glycogen synthesis occurred.

The greatest insulin effect, in terms of percentage increase, on glycogen deposition and on glucose uptake occurred with a glucose concentration of 2.0 mg per ml of medium. This finding agrees with that of Stadie and Zapp (6) but whereas they found that glycogen synthesis continued to increase as the glucose concentration increased up to 10.0 mg per ml, we found no increase occurring with a concentration greater than 4.0 mg per ml in the absence of insulin.

The duration of the incubation period affected both glycogen deposition and glucose uptake. It was found that while both responses increased during 90 minutes of incubation the difference was not significant between 60 and 90 minutes for glycogen synthesis. However, the greatest difference between control and insulin-treated tissues occurred at 90 minutes with glycogen deposition as the response. Since the conditions of the assay (1) require a change in glycogen concentration it is considered desirable to use the 90-minute incubation period for assay purposes.

Contrary to the findings of Brown *et al.* (7) the insulin effect was reduced if the tissues were chilled for 10 minutes or more. Krahl (10) has stated that the rate of glucose uptake by rat diaphragm over a 30-minute period depends upon the extent to which the tissues have been preincubated in chilled Krebs–Ringer bicarbonate medium. At least two published methods for insulin assay which measure glucose uptake by isolated rat diaphragm, those of Groen *et al.* (11) and Vallance Owen and Hurlock (12), have incorporated preincubation chilling as an integral part of their technique. The studies reported in Table V indicate that this step can be eliminated without prejudice to either response parameter.

Since muscle glycogenesis only proceeds within the intact cell, it is unlikely that this decreased glycogen deposition, as shown in Table V, would result from the leaching of requisite enzymes and coenzymes into the preincubation medium. Although it is possible that this mechanism, i.e. the loss of glycolytic enzymes and cofactors to the soaking fluid, could be responsible for the lower glucose uptake, this has no bearing on the assay procedure since all tissues are given identical treatment. In this regard, Randle and Smith (13) were unable to demonstrate significant glycolytic enzyme activity in the incubation fluid after the removal of the hemidiaphragms and limited unpublished studies from this laboratory support this finding.

Acknowledgments

We wish to thank Mr. John Carter for his excellent technical assistance.

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THE PARTIAL ELUCIDATION OF THE STRUCTURE OF A NEW COUMARIN FROM HYDRANGEA MACROPHYLLA SER.[1]

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Abstract

Umbelliferone and a new monohydroxymonomethoxycoumarin have been isolated from *Hydrangea macrophylla* Ser. The new coumarin has been shown to be a derivative of daphnetin. It is present largely in a bound form in all parts of the plant and is especially abundant in the roots. Chromatographic characteristics of the compound as well as those of several known coumarins are included in this report.

Introduction

Coumarins and coumarin derivatives are of widespread occurrence in plants, often accumulating in relatively large quantities. Umbelliferone (7-hydroxycoumarin) was reported to occur in the roots of *Hydrangea paniculata* and was isolated from this source by Hashimoto and Kawana (1). More recently, Nakahara (2) isolated neohydrangin from the inner bark of *H. paniculata*. Neohydrangin is a glycoside which yields umbelliferone and two molecules of glucose on hydrolysis. Apart from the isolation of hydrangenol (3) and a study of the anthocyanins (4) nothing is known of the phenolic compounds of *H. macrophylla*. It is the purpose of this paper to describe the detection, isolation, and partial elucidation of the structure of a new coumarin from *H. macrophylla*.

In a study of the biogenesis of the phenyl isocoumarin, hydrangenol (3), large fractions of the activity from various C^{14}-labelled precursors administered to various parts of the plant were observed to be in two unidentified phenolic compounds. The activity was of such a degree of intensity that it was considered of interest to identify the compounds.

Methods and Materials

Chromatographic Methods

The chromatographic techniques used in the present work have been described in detail in a previous publication (5).

Isolation of Compound "A"

Eight hundred grams of *Hydrangea* roots from plants grown in the greenhouse was washed with water to remove soil particles, blotted, and extracted in a Waring Blendor with boiling 85% ethanol. The ethanolic extract was

[1]Manuscript received May 4, 1961.
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[3]Present address: Botany Department, Faculty of Science, University of Alexandria, Egypt. This paper is taken, in part, from the Ph.D. Thesis of R. K. Ibrahim, McGill University, 1961.

filtered and the residue was further extracted with ethanol under reflux for 12 hours. In all, four portions of ethanol amounting to a total of about 4 liters were used. The combined extracts were reduced to a small volume under reduced pressure and further reduced to a thick syrup under an air jet. The syrup was dissolved in 300 ml of hot water and filtered with suction through a bed of Celite. The filtrate was hydrolyzed with 2 *N* hydrochloric acid under reflux for 2 hours. The acid hydrolyzate was continuously extracted with ether for 12 hours. The ether was removed by evaporation under reduced pressure and the residue dissolved in 85% ethanol. The ethanolic solution was applied to several sheets of Whatman No. 3 paper in the form of bands and chromatographed in benzene–acetic acid – water solvent (5). The bands corresponding to the compound were marked by their fluorescence, cut out, eluted with ethanol, and chromatographed using the formate solvent (5). Since eluates from formate chromatograms contain considerable amounts of sodium formate, the compound was further purified by continuous extraction with ether. The ether extract was reduced in volume and transferred to a semimicro sublimation apparatus and sublimed twice. The yield of white needles amounted to 65 mg.

Isolation of Compound "B"

The procedure for the isolation of compound "B" was essentially the same as that used for compound "A" described above, except that the extract was hydrolyzed first with base and then with acid. In order to free the compound from small amounts of contaminants, it was necessary to chromatograph the compound several times in each solvent system. Final purification was achieved by two sublimations. By this procedure two kilograms of fresh roots yielded 90 mg of fine white needles. Compounds "A" and "B" occur free, but larger quantities are available on acid or alkaline hydrolysis. Both compounds are present in roots, in smaller amounts in the leaves, and in trace quantities in the flowers.

Spectrophotometric Measurements

Ultraviolet spectra described in this work were taken with a Beckman Model DU ultraviolet spectrophotometer. Fused silica cells were used throughout.

The infrared spectrum was determined on a Baird atomic infrared spectrophotometer using sodium chloride optics. The sample was run as a KBr pellet.

Results and Discussion

The unknown compounds were both fluorescent under untraviolet light and gave color reactions with diazotized *p*-nitroaniline and diazotized sulphanilic acid. These color reactions are shown in Table I.

The white crystals of compound "A" from sublimation had a melting point of 225–226° (uncorr.). A mixed melting point of the unknown with an authentic sample of umbelliferone gave no depression (lit. m.p. of umbelliferone

TABLE I

Color reactions and fluorescence of unknown compounds

Compound	Fluorescence		Color reactions	
	U.V.	U.V., NH_3	PNA*	DSA†
"A"	Blue	Intensified	Blue (turns brown)	Pink (turns red)
"B"	Pale yellow–green‡	Yellow	Blue (turns brown)	Pink (turns red)

*Diazotized *p*-nitroaniline.
†Diazotized sulphanilic acid.
‡On formate chromatograms. When neutral, this compound exhibits a pale blue fluorescence. Under exposure to ultraviolet light, however, the color slowly changes to a pale yellow to yellow-green color.

223–224° (6)). Two-directional chromatography in two sets of solvent systems, benzene – acetic acid – water (6:7:3) followed by formate and butanol – acetic acid – water (4:1:2.2) followed by formate, gave only one spot when the isolated compound and known umbelliferone were chromatographed together. The fluorescent behavior, color reactions, and ultraviolet spectrum of the unknown compound matched exactly those of umbelliferone.

The similarity of the color reactions and fluorescent behavior of the two compounds led us to consider the possibility that unknown compound "B" might be a coumarin and might indeed be closely related to umbelliferone. Results from biogenetic experiments using C^{14}-labelled precursors further suggested that the two compounds were related. Considerable activity was found in the spots corresponding to "A" and "B" when the following compounds were fed: L-phenylalanine-U-C^{14}, L-phenylalanine-1-C^{14}, L-phenylalanine-3-C^{14}, sodium cinnamate-2-C^{14}, and shikimic acid-U-C^{14}. These experiments are to be described in a later publication.

That phenylpropanoid compounds are good precursors of coumarin has been demonstrated by Brown, Towers, and Wright (7). These workers showed that *o*-coumaric acid, cinnamic acid, shikimic acid, and phenylalanine were utilized by *Hierochloë odorata* in the formation of coumarin. Kosuge and Conn (8) have shown that the shikimic acid pathway functions in the biogenesis of coumarin in *Melilotus alba* Desr. Reznik and Urban (9) have reported the incorporation of ferulic acid in the biogenesis of scopoletin (6-methoxy-7-hydroxycoumarin) in *Triticum*, *Zea*, and *Helianthus* species.

In a further experiment, radioactive spots of umbelliferone were removed from two-directional paper chromatograms (from feedings mentioned above) and the compound eluted with ethanol. After removal of the solvent, the umbelliferone was removed from the residual sodium formate by continuous ether extraction. The ether solution was evaporated to dryness and the residue was taken up in a small volume of water. This aqueous solution of radioactive umbelliferone was administered to leaf disks of *Hydrangea*. After 16 hours the leaf disks were extracted with ethanol, the extract was reduced to dryness, taken up in hot water, acidified, and extracted continuously with ether. The

extract was chromatographed in the benzene–formate solvent systems. Radioautography showed residual umbelliferone and an area of activity corresponding to the position of the unknown compound "B". It was clear from this experiment that umbelliferone was readily incorporated into compound "B".

The needles of "B" obtained on sublimation had a melting point of 157–157.5°. The compound had a rather pleasant odor resembling that of coumarin. Carbon–hydrogen analysis of a sample gave the following percentages: C, 62.8%; H, 4.26%. An infrared spectrum of the compound was obtained and was found to be consistent with a molecule containing a lactone, a free hydroxyl group, a methyl group, and an ether linkage. The latter two functional groups may suggest the presence of a methoxyl substituent. The absence of a free carboxyl function was also demonstrated by the compound's failure to give a color reaction on chromatograms when sprayed with bromcresol green acid–base indicator solution.

The ultraviolet spectrum of compound "B" was virtually identical with that of umbelliferone in neutral and alkaline solution. Both compounds exhibited maxima at 325 mμ when run in 95% ethanol which shifted to 377 mμ upon the addition of two drops of 2 *N* sodium hydroxide. It has been reported (6) that substituted coumarins have absorption maxima in this range.

Treatment of "B" with acetic anhydride in anhydrous pyridine for 18 hours gave a new compound which had a melting point of 134–134.5°. Recrystallization of this compound from water gave white silky needles with the same melting point.

Treatment of compound "B" with hot, dilute sodium hydroxide solution followed by careful neutralization with dilute acid in the cold gave a compound which gave two fluorescent spots when chromatographed in the formate solvent. This suggests the presence of cis and trans isomers of a substituted cinnamic acid. Reduction of the open ring derivative with hydrogen and palladium on carbon (45 p.s.i.) gave a compound which produced only one spot on chromatography in the formate solvent. This compound was not fluorescent and gave color reactions differing from those of both the unknown compound "B" and its open ring derivative. These results are in line with the presence of a reducible double bond as would be found in cinnamic acid derivatives.

Data shown to this point suggest a monohydroxymonomethoxycoumarin. The only monohydroxymonomethoxycoumarin known to occur naturally in plants is scopoletin. Figure 1 shows that compound "B" and scopoletin are chromatographically separable. Moreover, the melting point of the unknown does not correspond to that of any other recorded coumarin. Melting points of some pertinent compounds are shown in Table II. Two of the possible dihydroxycoumarins are well known as naturally occurring compounds. These are the 6,7- and 7,8-dihydroxycoumarins (esculetin and daphnetin, respectively). The 5,7-dihydroxy compound has also been reported in the chemical literature (10). Removal of the methyl group from the ether linkage

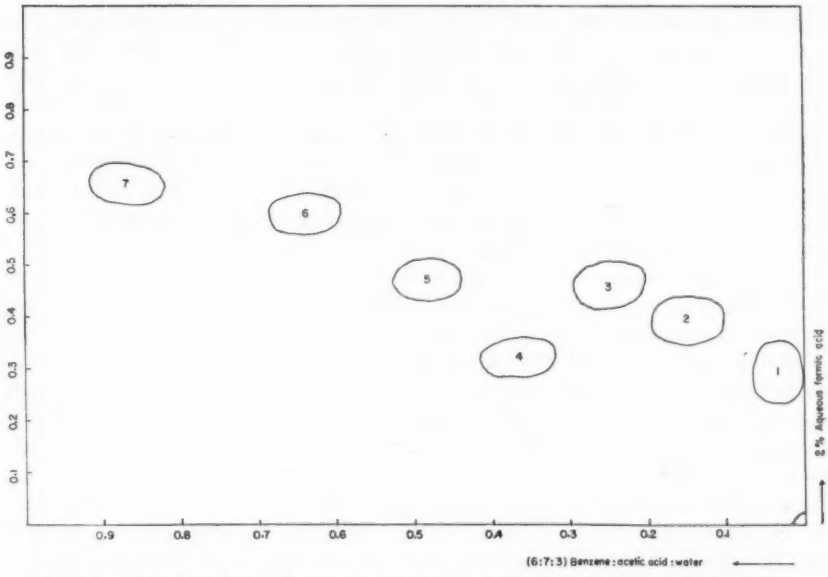

FIG. 1. Two-directional chromatogram of some coumarins. Identification: (1) esculetin, (2) daphnetin, (3) umbelliferone, (4) scopoletin, (5) "iso-B", (6) unknown "B", (7) 7,8-dimethoxycoumarin.

TABLE II

Melting points of some common coumarins

Compound	Trivial name	Melting points	Ref.
7-Hydroxycoumarin	Umbelliferone	223–224°	6
7-Methoxycoumarin	Herniarin	117–118°	12
5,7-Dihydroxycoumarin	—	285–286°	10
6,7-Dihydroxycoumarin	Esculetin	268–270°	13
7,8-Dihydroxycoumarin	Daphnetin	255–256°	11
6-Methoxy-7-hydroxycoumarin	Scopoletin	203–204°	14
6-Hydroxy-7-methoxycoumarin	—	184°	15
5,7-Dimethoxycoumarin	Limettin	148°	16
6,7-Dimethoxycoumarin	—	144°	17
7,8-Dimethoxycoumarin	—	119–121°	18

of "B" might then result in the formation of one of these known compounds. The demethylation was accomplished by heating the ether with concentrated hydriodic acid in the usual manner. The product was removed from the reaction mixture by ether extraction and the resultant solid purified by chromatography in the benzene – acetic acid – water solvent followed by sublimation. This procedure gave a white product which melted with considerable sublimation at 250–260°. The melting point of 7,8-dihydroxycoumarin is recorded in the literature as 255–256° (11). An authentic sample of 7,8-dihydroxycoumarin was synthesized by the Pechmann reaction (11)

between pyrogallol and malic acid in the presence of sulphuric acid. The compound prepared in this way melted, with sublimation, at 250–260°. The chromatographic behavior of "demethyl-B" and of 7,8-dihydroxycoumarin were identical in all respects. Their color reactions with diazotized-*p*-nitroaniline and diazotized sulphanilic acid as well as their fluorescent characteristics were also identical. The ultraviolet absorption spectra of "demethyl-B" and 7,8-dihydroxycoumarin were identical, with a sharp peak at 262 mμ and a broader peak at 325–328 mμ. Upon the addition of alkali the major peak in each compound shifted to 406 mμ.

Partial methylation of daphnetin with dimethyl sulphate gave a mixture of three products which were separable by paper chromatography. These compounds, whose positions are shown on the map in Fig. 1, were the two hydroxymethoxycoumarins and the dimethoxycoumarin. The isomeric hydroxymethoxycoumarin whose spot matched that of compound "B" had properties corresponding exactly to those of the unknown. The melting point of the synthetic "B" was 153–155°, while that of its isomer was 170–173°. With diazotized sulphanilic acid "iso-B" gave a pink color which rapidly turned brown; with diazotized *p*-nitroaniline it gave a brown spot with a violet border which also turned brown. Under neutral conditions the compound fluoresces a dull blue-grey. When the spot is exposed to ammonia vapors the color changes to a dull olive. "Iso-B" showed absorption maxima (95% EtOH) at 263 mμ and 320 mμ. These peaks shifted to 279 mμ and 329 mμ upon the addition of two drops of 2 *N* NaOH solution.

From the foregoing, the possible structures may be reduced to two: 7-hydroxy-8-methoxy- and 7-methoxy-8-hydroxy-coumarin. Evidence which supports the 7-hydroxy-8-methoxy structure is as follows: (*a*) the incorporation of umbelliferone in the biosynthesis of the unknown; (*b*) the unknown and umbelliferone exhibited essentially identical ultraviolet spectra and color reactions; and (*c*) the unknown compound gave a bright fluorescence under ultraviolet light whereas the isomer gave only a very dull color under the same conditions. This last point is in line with the conclusions of Goodwin and Kavanagh (19) on the fluorescence characteristics of coumarin derivatives. However, the final assignment of structure must await the development of an unequivocal synthesis of the unknown. It should be emphasized that the bulk of this compound occurs in a bound form in the plant, possibly as a glycoside. No attempts, however, have been made to isolate this compound.

Acknowledgments

We wish to thank the National Research Council of Canada for financial support of this work. The assistance of Dr. Paul I. Abell of the University of Rhode Island in the determination of the infrared spectrum is greatly appreciated. Thanks are also due to Dr. V. Runeckles of Imperial Tobacco Ltd. and Dr. W. Andreae of the Canada Department of Agriculture for samples of scopoletin.

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DETERMINATION OF SOLUBLE CARBOHYDRATES FROM THE ABSORBANCES OF SULPHONATED 1-NAPHTHOL DERIVATIVES OF HEXOSES AND PENTOSES[1]

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Abstract

A modified Molisch test was used in quantitative spectrophotometric analyses for glucose and xylose in mixed solutions. The *algebraic method* for determination of substances exhibiting additive absorbances was found to apply to these mixtures. The two wavelengths at which the most reliable measurements were obtained were 530 and 580 mμ. An isosbestic point was found at 562.7 mμ at which the glucose–xylose ratio had no effect on the absorptivity, thus allowing a measurement of the sum of the concentrations of the two sugars.

This method of analysis was used to determine the concentrations of hexoses and pentoses in acid extracts of forages and feces from sheep fed these forages in digestibility experiments. The carbohydrates detected by this method of analysis had a greater digestibility than is usual for the conventional *nitrogen-free extracts*. The digestibilities of hexoses and pentoses extracted from several forages averaged 87 and 70%, respectively. The levels of uronic acids in the extracts were found to be below the level of appreciable interference with the measurements of the monosaccharides.

Introduction

Devor (1, 2, 3) modified the Molisch test from a qualitative test to a quantitative measure of soluble carbohydrates. Presulphonation and crystallization of 1-naphthol yielded sulphonated 1-naphthol crystals and a dark-colored substance (1). The reaction products of an aqueous solution of these crystals with hexoses and pentoses exhibited a spectral absorption conforming to Beer's law (2). A common absorption band with a maximum near 570 mμ* existed for all the hexoses, while a common band for the pentoses showed maximum absorption near 550 mμ (3). As no interfering substances were found in acid extracts of various cereal products (4, 5), the reaction provided a quantitative measure for the soluble hexosans and pentosans. A glucose standard was shown to be adequate in determining the concentrations of hexoses and pentoses in mixtures where pentoses constituted less than 14% of the monosaccharides (4). This report shows an application of the *algebraic method* for determination of substances exhibiting additive absorbances (6) in estimating the concentrations of hexoses and pentoses when mixed in any proportions. The analysis was also applied to extracts of several forages and feces from sheep fed these forages in digestibility experiments.

Materials and Methods

Sulphonated 1-naphthol crystals were prepared from 1-naphthol and 95.5% H_2SO_4 as recommended by Devor (1). Ten grams of crystals was dissolved in

[1]Manuscript received January 6, 1961.
Contribution from the Research Branch, Canada Department of Agriculture, Experimental Farm, Swift Current, Saskatchewan.

*The nomenclature used is that proposed by the A.O.A.C. Committee on spectrophotometry (Journal of Official Agricultural Chemists, **37**, 54 (1954)).

100 ml of water and stored in dark-glass bottles at 5° C. This solution is referred to as the reagent. Standard aqueous solutions of monosaccharides (referred to as standards) were prepared from anhydrous D-glucose, purified D + xylose, and mixtures of these. The sulphuric acid extracts, from the crude fiber analyses (referred to as extracts) of the forage and feces samples, were made up to known volumes. At the time of analysis, extracts were diluted to contain approximately 20 to 50 mg of monosaccharides per liter to comply with the range of the spectrophotometer.

The color was developed by adding 2 ml of reagent and 10 ml of 95.5% H_2SO_4 to 2 ml of standard or extract in a 50-ml test tube. The mixture was vigorously agitated for 30 seconds and immersed in a boiling-water bath for 8 minutes. The samples were held in an ice bath until the spectrophotometric readings were taken. A Beckman DU spectrophotometer with 1-cm cuvettes was used. Times and temperatures were those recommended by Devor (4).

Results and Discussion

The reaction products of sulphonated 1-naphthol with a mixture of glucose and xylose were found to conform to Beer's law (6) as verified by a rectilinear relationship between the concentration and absorbance (A) at each of the wavelengths illustrated in Fig. 1. A section of the spectral absorbance curves covering the major absorption bands of the reaction products of 11 different glucose–xylose mixtures of equal total concentration is illustrated in Fig. 2.

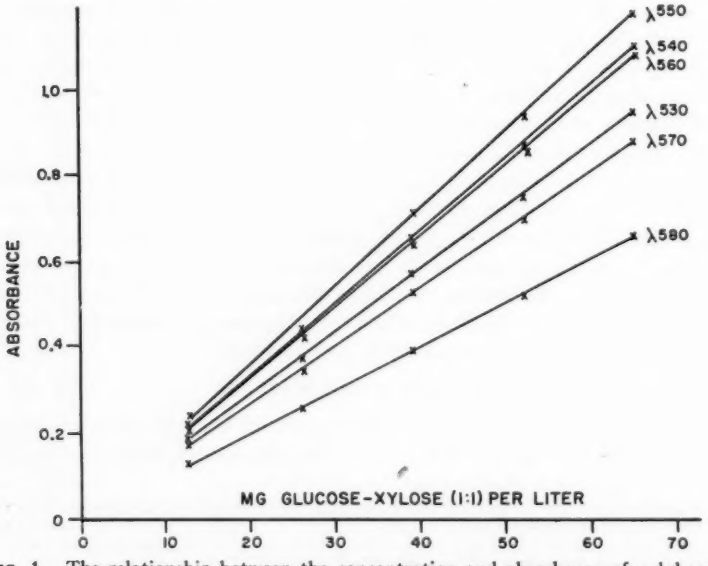

FIG. 1. The relationship between the concentration and absorbance of sulphonated 1-naphthol reaction products with a glucose–xylose mixture.

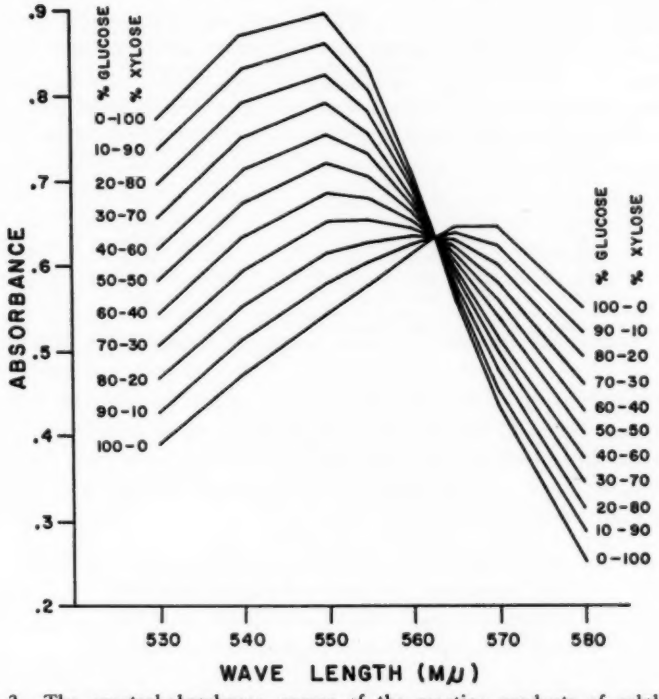

FIG. 2. The spectral-absorbance curves of the reaction products of sulphonated 1-naphthol with 11 different mixtures of glucose and xylose, at a total concentration of 40 mg per liter.

The relationships between the absorbance (X) and the glucose fraction (Y) have been expressed by the regression equations ($Y=a+bX$) calculated for each of seven wavelengths by the method described by Snedecor (7, page 127). The calculated values are presented in Table I. The *goodness of fit* of the data to the theoretical line expected from the assumption of additive absorbances is indicated by the *standard deviation* from this line (Table I), and reflects the reliability of a rectilinear relationship between the glucose fraction and the absorbance of glucose–xylose mixtures. The magnitude of the *standard deviations* warranted the assumption that the absorbances of glucose and xylose were additive according to the test described by Stearns (6, page 373). When this condition is fulfilled the *algebraic method* described by Stearns (6, page 374) may be used to determine the concentrations of the two components in unknown mixtures.

The two wavelengths (λ_1 and λ_2) giving the greatest accuracy in the analysis were determined as shown by Stearns (6, page 369). Because of the low absorption of glucose below 530 mμ and of xylose above 580 mμ (Fig. 2), it was considered advisable to eliminate from consideration the areas of the

TABLE I

The relationship between absorbances and glucose fractions in glucose–xylose mixtures of known compositions (Total concentration 40 mg per liter)

Wavelength (mμ)	Regression equations ($Y=a+bX$)*	The standard deviation from the theoretical line†
530	$Y=0.771-0.379X$	.016
540	$Y=0.875-0.408X$	.017
550	$Y=0.898-0.354X$	.020
560	$Y=0.710-0.096X$	.023
565	$Y=0.566+0.080X$	.022
570	$Y=0.436+0.210X$	.018
580	$Y=0.260+0.291X$	.017

*Y = absorbance; X = fraction of glucose.
†Calculated on the assumption of additive absorbances of glucose and xylose derivatives.

spectral absorbance curves outside these wavelengths. In doing so, λ_1 was found to be 580 mμ and λ_2 to be 530 mμ. The absorbances of the glucose and xylose standards at these wavelengths ($A\lambda_1$ and $A\lambda_2$) were obtained from the respective regressions in Table I by substituting the fractions of glucose (one and zero) for X in each equation as follows:

$$A\lambda_1 \text{ for glucose} = 0.260+0.291\times1 = 0.551,$$
$$A\lambda_1 \text{ for xylose} = 0.260+0.291\times0 = 0.260,$$
$$A\lambda_2 \text{ for glucose} = 0.771-0.379\times1 = 0.392,$$
$$A\lambda_2 \text{ for xylose} = 0.771-0.379\times0 = 0.771.$$

These absorbances were used in the conventional manner (6, page 374) for calculating the constants to be used in equations 1 and 2 to determine the unknown concentrations of hexoses and pentoses. When these concentrations were expressed as milligrams per liter and the absorbances of the extracts were designated $Ae\lambda_1$ and $Ae\lambda_2$, the equations could be written

[1] $$\text{mg of hexoses per liter} = (94.93\times Ae\lambda_1)-(31.63\times Ae\lambda_2),$$
[2] $$\text{mg of pentoses per liter} = (67.78\times Ae\lambda_2)-(47.96\times Ae\lambda_1).$$

The constants in equations 1 and 2 cannot be expected to apply to other spectrophotometers than the one used for this work. In determining the constants for other instruments it is suggested that the calculation may be based upon the A's of the two pure sugars at λ_1 and λ_2.

The regressions in Table I also revealed that at some wavelength between 560 and 565 mμ, the coefficient of X would be zero. This wavelength (λ_i) would be an isosbestic point on the spectral absorbance curve (Fig. 2) where the ratio of glucose and xylose would have no effect on A. Consequently, the total concentration of monosaccharides (hexoses + pentoses) could be determined from A at λ_i. By interpolation between λ_{560} and λ_{565} mμ and the corresponding regressions in Table I, λ_i was estimated to be at $\lambda_{562.7}$ mμ; this is illustrated in Fig. 2 at the point where the curves intercept each other.

Since the *algebraic method* of Stearns (6) was applicable to the absorbances of glucose–xylose mixtures, it should also apply to mixtures of hexoses and pentoses in general (Devor (3)). Although the possibility of interfering uronic acids (8), particularly in the feces samples, could not be ruled out, extracts of forages and feces from sheep fed these forages were analyzed by this method. The absorbances of the colors developed by the extracts were measured at the same wavelengths between 530 and 580 mμ as had been the colors for the standard mixtures. The values were reduced so as to relate to a concentration of 40 mg/liter of monosaccharides and averaged for each wavelength. In Fig. 3 the averaged spectral absorbance curve is compared to the

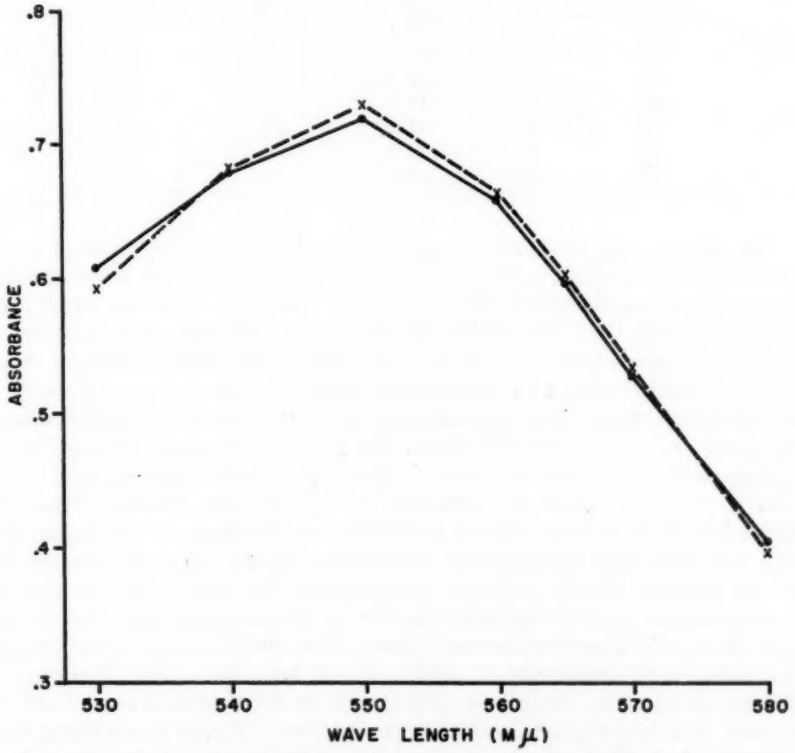

FIG. 3. The averaged spectral-absorbance curves of the reaction products of sulphonated 1-naphthol with monosaccharides in extracts of forages and corresponding feces (——) compared to the corresponding curve for a known glucose–xylose mixture (– – –), total concentration 40 mg per liter.

standard glucose–xylose curve for a similar hexose fraction (0.47) calculated from the equations in Table I. The similarity of the two curves indicates that appreciable amounts of interfering substances could not have been present in

the extracts unless such substances possessed absorption patterns resembling those of hexoses and pentoses. The absorptivity (a) data in Table II show no such resemblance between monosaccharides and uronic acids which suggests that the amounts of the latter in the extracts were negligible.

TABLE II

Absorptivity (a) of sulphonated 1-naphthol derivatives

	absorptivity (a) of:			
Wavelength (mμ)	Glucose (A)	Xylose (A)	Glucuronic acid (A)	Galacturonic acid (A)
530	9.8	19.3	4.6	3.9
540	11.7	21.9	4.2	3.8
550	13.6	22.5	3.9	3.6
560	15.4	18.0	3.7	3.4
565	16.2	14.2	3.4	3.2
570	16.2	10.9	3.0	3.0
580	13.8	6.5	3.0	2.7

The forages and feces analyzed are listed in Table III. The levels of monosaccharides determined in the extracts of these were converted to percentages of soluble polysaccharides in the dry matter. This conversion was based upon the assumption that the soluble hexosans and pentosans were completely degraded to monosaccharides prior to, or during, the Molisch reaction. Since the samples originated from digestibility trials with sheep, it was possible to calculate digestibility coefficients for the two groups of soluble carbohydrates. The bottom line of Table III shows the difference required for significance between any pair of values within a column (7). Some characteristics of the data in this table show the usefulness of the analyses outlined. Thus, the rather low levels of total soluble carbohydrates (hexosans + pentosans) and their relatively high digestibilities as compared to the values for *nitrogen-free extracts* generally found in forage crops, suggest that this method of analysis is determining the more digestible fraction of the carbohydrates. Indeed, the digestibility of the soluble hexosans approaches 100% in some of the forages. The digestibility coefficients of pentosans and hexosans support the concept (8) that pentoses are less readily absorbed from the intestinal tract than are hexoses. It is interesting to notice that in the three different lots of fescue hay, *Festuca scabrella Torr.*, the levels of soluble pentosans were higher than those of hexosans. The high level of soluble hexosans in western wheatgrass, *Agropyron smithii* L., is interesting in view of the fact that this species is regarded as one of the more nutritious native hays.

The method of analysis described in this report is presently being used in this laboratory to study the relative nutritive value of hexosans and pentosans in forage crops.

TABLE III

Carbohydrates dissolved in the acid extracts from the crude fiber analyses of forages and feces from sheep fed these forages in digestibility experiments

Forages	% of dry matter in:				% digestibility	
	Forage		Feces			
	Hexosans	Pentosans	Hexosans	Pentosans	Hexosans	Pentosans
Oat hay	14.5	5.3	2.1	5.8	94	48
Oat hay (dried at 100° C)	14.9	5.3	1.9	5.5	93	48
Western wheatgrass hay	11.9	5.2	2.5	3.1	89	66
Fescue hay No. 1	6.6	6.9	2.1	3.5	83	77
Fescue hay No. 2	5.3	7.1	2.0	3.6	80	73
Alfalfa hay	6.1	3.1	1.2	1.8	91	78
Alfalfa hay + Fescue hay No. 3 (2:1)	5.6	4.5	2.1	2.7	86	75
Alfalfa hay + Fescue hay No. 3 (1:2)	5.2	6.5	2.3	3.3	82	78
Fescue hay No. 3	5.2	8.9	2.5	5.0	78	73
Alfalfa hay + crested wheatgrass hay	9.3	4.3	2.4	2.2	91	83
Difference ($P < 0.05$)*	1.2	0.5	0.6	0.6	3	4

*The difference required for significance at $P < 0.05$ (7).

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MOLECULAR WEIGHT OF BOVINE PLASMA ALBUMIN, VITELLENIN, AND THEIR OXIDATION PRODUCTS IN FORMIC ACID[1]

W. G. MARTIN

Abstract

Sedimentation, diffusion, and Archibald transient state measurements were made on bovine plasma albumin and vitellenin of egg yolk in formic acid (88% w/w) solution. The molecular weight of bovine plasma albumin, averaging 77×10^3 and 70×10^3 with and without added salt, respectively, indicated that peptide bonds were stable to the acid for at least 1 week (storage at 5° C and measurement periods at 20° C). Similar values were obtained from estimates based on viscosity measurements but greater deviations occurred. Vitellenin had a mean molecular weight of 93×10^3 from sedimentation and diffusion but polydispersity was revealed by the Archibald measurements (molecular weights from 55×10^3 to 10×10^3). Higher values of molecular weight were obtained for vitellenin by varying the dissolution technique and exposure time in formic acid and also when viscosity measurements were used to compute molecular weight. Analyses of N-terminal amino acids showed that peptide bond rupture was not a major factor in the polydispersity of vitellenin. Although aggregates are probably present in formic acid solutions of this protein, it appears to be naturally polydisperse. Both albumin and vitellenin were considerably degraded by performic acid oxidation procedures.

Introduction

In a recent study (1) of the macromolecular properties of vitellenin, the protein of the low-density fraction of egg yolk, 88% formic acid was used as the solvent because the lipid-free protein was insoluble in mild aqueous solutions. Concentrated formic acid appeared to be suitable since active enzymes have been recovered following dissolution in this solvent (2, 3, 4), and it had more favorable physical properties than other organic acids with the necessary solvent power. Except for a rather high estimate of the size of bovine serum albumin in formic acid by light-scattering techniques (5), measurement of the molecular weight of proteins in this solvent lacks precedent.

Molecular weight determinations were therefore undertaken on bovine plasma albumin (a protein of known size) in 88% formic acid by the Archibald procedure (6) and from sedimentation, diffusion, and viscosity measurements. Similar measurements were made on vitellenin and on both proteins after oxidation with performic acid. N-Terminal amino acid analyses of vitellenin were also made before and after exposure to formic acid.

Materials and Methods

Crystallized bovine plasma albumin (BPA) was used as purchased (Armour and Company Lot No. 68204). Vitellenin was prepared as described by

[1]Manuscript received May 1, 1961.
Contribution from the Division of Applied Biology, National Research Council, Ottawa, Canada.
Issued as N.R.C. No. 6437.

Martin *et al.* (1). Unless stated otherwise, the formic acid used as solvent was 88% (w/w) and is subsequently referred to as formic acid. Routinely the proteins were dissolved in formic acid by stirring with a magnetic stirrer at room temperature for 3 to 4 hours and then stored at 5° C between measurements. Physical measurements on the unoxidized proteins were made within 1 week of dissolution in formic acid except when extended exposure time is mentioned in the text.

Concentration (c) was determined by dry weight or by specific refractive index increment (dn/dc) calibrated by dry weight (7). Measurement of density (ρ) was made in a magnetic float apparatus (8) for computation of partial specific volume ($\bar{v}$) and with a Mohr–Westphal specific gravity balance for other than $\bar{v}$ determinations. Diffusion measurements were made by schlieren optical methods in a Spinco Model E ultracentrifuge; and viscosity, in Ostwald–Fenske capillary viscometers. Conductivity measurements were made on formic acid, a dialyzed solution of 1% vitellenin in formic acid and its diffusate. Potassium chloride solutions were used as standards.

BPA and vitellenin (final protein concentration 0.4 to 2%) were oxidized by performic acid as described below:

(*a*) The oxidation mixture was 1 volume of 30% H_2O_2 plus 5 volumes of formic acid. During a reaction time of 25 minutes in a room at 25° C the temperature of the BPA solution increased substantially (above 40° C), while the vitellenin solution remained at 25° C. The performic acid was removed by dialysis versus changes of formic acid at 5° C.

(*b*) The procedure was similar to (*a*) except that the temperature of the BPA solution was not permitted to exceed 40° C during the reaction.

(*c*) The cooled oxidation mixture of 1 volume of 30% H_2O_2 plus 11 volumes of formic acid reacted with the protein in a room at −10° C for 2½ hours. The performic acid was then removed by dialysis versus changes of formic acid at 5° C.

(*d*) The procedure was similar to (*c*) except that the oxidation occurred in an ice bath at 0° C, dialysis was versus water, and the final step was lyophilization.

Difficulties imposed by the low sedimentation and rapid diffusion rates of the oxidized proteins and the corrosive action of the solvent, formic acid, were overcome by modifying a standard Kel-F centerpiece to form synthetic boundaries in a manner somewhat similar to that described by Kegeles (9). Two blind holes, 3 mm in diameter, were drilled from the same face of the centerpiece to serve as solvent reservoirs, one on each side of the narrower end of the open sector. A shallow groove was made across the face of the centerpiece from the top of each reservoir to the top, and from the bottom of each reservoir to the middle, of the sector-shaped opening, considering the top as being closest to the center of rotation. This permitted solvent to leave, and air to enter, both reservoirs under centrifugal force. The face of the plastic centerpiece was then ground to remove the annular ridge and polished to

prevent leakage. The rate of solvent emission was adjusted by face grinding to reduce the depth of the grooves until sharp boundaries were formed between solvent and solution at speeds between 5,000 and 10,000 r.p.m.

Experimental procedure with the modified cell consisted in filling the two reservoirs of the centerpiece of the partially assembled cell, assembly of the cell, and tightening of the cell by hand, all in a horizontal position. Final tightening was then made in a bench vise and solution added to the cell.

Apparent molecular weights, M_{app}, were obtained by a modification of Trautman's treatment (10) of the Archibald relation (6). Values of $(1/\omega^2 r_m)$ (dn/dr_m) as ordinates were plotted against corresponding values of

$$1/r^2_m \int_{r_m}^{r_p} r^2 \, (dn/dr) dr$$

as abscissas, where ω and r are the angular velocity and distance from the center of rotation, subscripts m and p refer to the meniscus and plateau region of the solution in the ultracentrifuge cell, and the quantity included in the integral is the second moment of the schlieren pattern about the axis of rotation. The slope $-(M_{app}\,(1 - \bar{v}\rho)/RT)$ of the lines joining each of these points to n_0, the refractive index difference between the original solution and solvent on the abscissa axis, permitted calculation of M_{app}. Values of c_m (concentration at the meniscus which varies with time) were calculated on the basis of the expression given by Klainer and Kegeles (11). The second moments of the schlieren patterns involved in these procedures were evaluated by means of a mechanical integrator from traced enlargements by the method of Smith *et al.* (12).

N-Terminal amino acid analyses were made by the 1-fluoro-2,4-dinitrobenzene (FDNB) method of Sanger (13). Manipulation and identification of the ether-soluble dinitrophenol (DNP) amino acids of the acid hydrolyzates were made as described by Smith and Turner (14). The ether-extracted aqueous phase was freed of amino acids and salts by chromatography on talc (15) after which a second reaction with FDNB was performed. After another ether extraction the aqueous phase was examined for DNP amino acids. The aqueous phase was also examined before talc column chromatography by paper chromatography and paper electrophoresis (16).

Results

A summary of the measurements made on BPA and oxidized BPA is given in Table I. Although the specific refractive index increments of BPA and oxidized BPA are the same within experimental error, both values are considerably lower than those observed in aqueous media. The higher refractive index of formic acid could account for most of this decrement in dn/dc. In formic acid BPA had $\bar{v} = 0.734$. The value of 0.717 for $\bar{v}$ of oxidized BPA is quite reasonable because calculation by the method of Traube (17), based upon the addition of 34 SO_3H groups to the protein upon oxidation, results in a value of 0.716 ml/g.

TABLE I

Summary of measurements of bovine plasma albumin in formic acid (88% w/w)

Property	BPA		Oxidized BPA*		
			Formic acid + 0.1 *M* NaCl		
	Formic acid	Formic acid + 0.1 *M* NaCl	(*a*)	(*b*)	(*c*)
$dn/dc \times 10^3$ at 5780, dl/g	1.48		1.47		
$\bar{v}20$, ml/g	0.734		0.717		
$[\eta]_{20}$, dl/g	0.318	0.216	0.069	0.105	0.159
$D^\circ_{20,w} \times 10^7$, cm²/sec	3.40	3.26	8.43		
$s^\circ_{20,w}$, Svedbergs	2.78	2.78	0.84	0.89	1.05
$M \times 10^{-3}$					
From s and D	75	78	8.6		
From $[\eta]$† and s	84	69	5.7	8.7	11.9
From $[\eta]$ and D	51	84	15.4		
From Archibald procedure ($c_m \rightarrow 0$)	79	62	12.2	16.0	10.5

*For performic acid oxidation procedures (*a*), (*b*), (*c*), see text.
†All M values from $[\eta]$ by (26) assuming $\beta = 2.23 \times 10^6$.

The effects of protein concentration on the sedimentation and diffusion coefficients and on the reduced viscosity of BPA are shown in Fig. 1. As the plots of s versus c for BPA were not linear, only $1/s$ vs. c plots are shown (Fig. 1A). This plot for the protein in formic acid alone was also curved but the intercept indicated the same sedimentation coefficient as the linear relation obtained in the presence of salt. The diffusion coefficients were also affected by the addition of salt to the formic acid, since concentration dependence was absent in formic acid + 0.1 *M* NaCl. Although the intrinsic viscosity ($[\eta]$) of BPA in formic acid was lowered by the addition of salt, it remained much higher than in aqueous solution (18).

The more reliable procedures (s and D, Archibald) indicate that the molecular weight of BPA in formic acid + 0.1 *M* NaCl is about 70×10^3 (Table I). This is similar to the value obtained in aqueous solvent. Oxidation greatly altered the physical properties of BPA and all the changes were in the direction of a reduced molecular size (19).

The results of the Archibald sedimentation experiments on BPA, oxidized BPA, and a 1:1 mixture of these in formic acid, with and without salt, are given in Fig. 2. As M_{app} of BPA varied with the elapsed sedimentation time, apparent molecular weights, calculated at selected intervals of time, were plotted against c_m (6, 20). For BPA in formic acid (Fig. 2A) this relation was not linear and therefore empirical corrections were made to each value of M_{app}, based upon the concentration dependence shown by the relevant sedimentation and diffusion in Fig. 1. The correction did improve the linearity and it decreased the concentration dependence. The intercepts from the uncorrected and the corrected curves were similar. The addition of 0.1 *M* NaCl to formic acid (Fig. 2B) reduced the degree of concentration dependence and gave a linear relation within the range of available measurements. Correction

for the concentration dependence of s and D decreased the slope and increased the value of the intercept. The M_{app} vs. c_m relations were linear (Fig. 2C) for oxidized BPA in formic acid + 0.1 M NaCl, and showed relatively little concentration dependence. The oxidation procedures appear to be equally degradative, considering the error of the Archibald measurements.

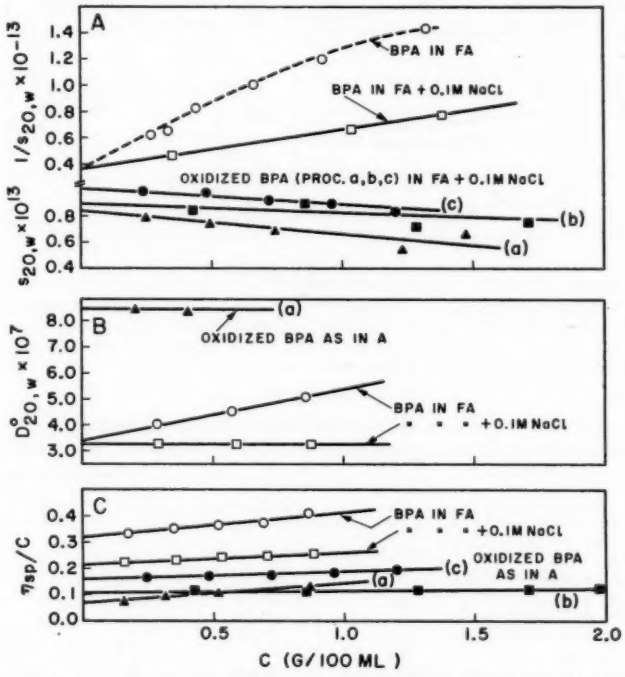

FIG. 1. Hydrodynamic behavior of bovine plasma albumin (BPA) and oxidized BPA in 88% w/w formic acid (FA) as a function of concentration. Oxidation procedures (*a*), (*b*), (*c*), as in text. (A) Sedimentation. (B) Diffusion. (C) Reduced viscosity.

To evaluate the qualitative effects of polydispersity a solution of a 1:1 mixture of BPA and oxidized BPA (procedure (*a*)) in formic acid + 0.1 M NaCl was examined and the results are plotted in Fig. 2D. Evidently, when the concentration dependence is within reasonable limits (Figs. 2B and 2C), the M_{app} vs. c_m plot reflects polydispersity and provides a qualitative indication of this condition.

Measurements on vitellenin and oxidized vitellenin are summarized in Table II. The $s_{20,w}$ vs. c plots (Fig. 3) were reproducible for different preparations of vitellenin, they were all linear, and more concentration dependent than for oxidized vitellenin. The presence of salt in low concentrations caused a small increase in the sedimentation coefficients but had no further effect at concentrations above 0.1 M NaCl.

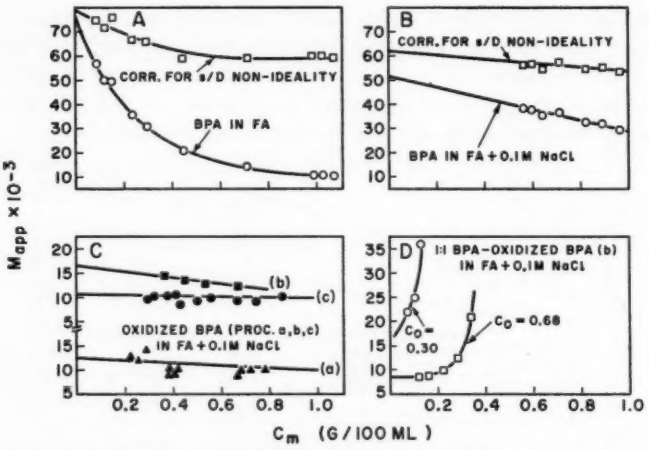

FIG. 2. Apparent molecular weight of bovine plasma albumin and oxidized bovine plasma albumin in 88% w/w formic acid (FA) as a function of the concentration of protein at the meniscus (c_m). (A) BPA in FA. (B) BPA in FA + salt. (C) Oxidized BPA + salt. (D) 1:1 BPA – oxidized BPA in FA + salt.

TABLE II

Summary of measurements on vitellenin in formic acid (88% w/w)*

Property	Vitellenin	Oxidized vitellenin†		
		(*a*)	(*c*)	(*d*)
$s^\circ_{20,w}$, Svedbergs				
Formic acid	2.39			
Formic acid + 0.05 *M* NaCl	2.54			
Formic acid + 0.10 *M* NaCl	2.75	0.87	0.87	1.84
Formic acid + 0.20 *M* NaCl	2.80			
Formic acid + 0.30 *M* NaCl	2.66			
$D^\circ_{20,w} \times 10^7$, cm²/sec				
Formic acid	2.49			
Formic acid + 0.2 *M* NaCl	(2.86)			
$[\eta]_{20}$, dl/g				
Formic acid + 0.10 *M* NaCl	0.88	0.101	0.105	0.390
$M \times 10^{-3}$ from coefficients‡				
s and *D*, formic acid	89			
s and *D*, formic acid + 0.2 *M* NaCl	(93)			
s and $[\eta]$, formic acid + 0.1 *M* NaCl	146	8.8	9.0	52
$M_{app} \times 10^{-3}$, Archibald procedure				
Formic acid	10 to 55			
Formic acid + 0.10 to 0.20 *M* NaCl	22 to 55	6.0		

*Values applicable to vitellenin exposed to formic acid < 7 days.
†For performic acid oxidation procedures (*a*), (*c*), (*d*), see text.
‡All *M* values from $[\eta]$ by (26) assuming $\beta = 2.23 \times 10^6$.
Values in parentheses from (1).

The diffusion coefficient was concentration dependent in formic acid (ca. 1.1×10^{-7} cm² sec^{-1}/%) with an intercept of 2.49×10^{-7} cm² sec^{-1}. This is lower than the 2.86×10^{-7} previously reported (1) for vitellenin in formic acid + 0.2 *M* NaCl where no concentration dependence was evident.

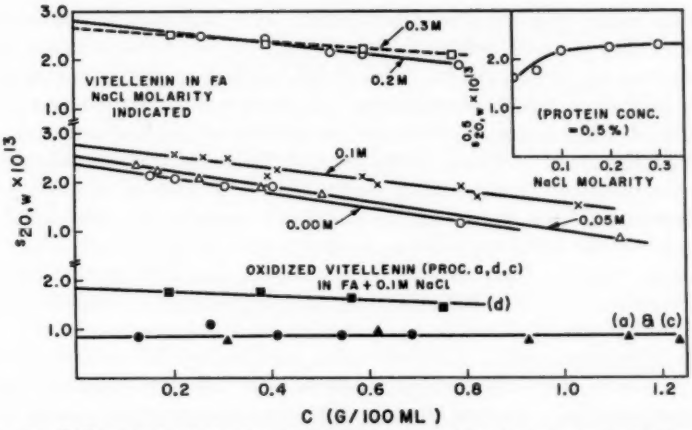

FIG. 3. Sedimentation behavior of vitellenin and oxidized vitellenin (oxidation procedures (*a*), (*d*), (*c*), as in text) in 88% w/w formic acid (FA) as a function of concentration. Insert: sedimentation of vitellenin as a function of salt concentration.

Reduced viscosity of vitellenin was markedly dependent on the salt concentration (Fig. 4) and was still decreasing at 0.3 *M* NaCl, the upper limit employed owing to the decreasing solubility of both salt and protein. All plots of vitellenin and oxidized vitellenin were linear. Clearly, oxidized vitellenin has a lower $[\eta]$ than vitellenin.

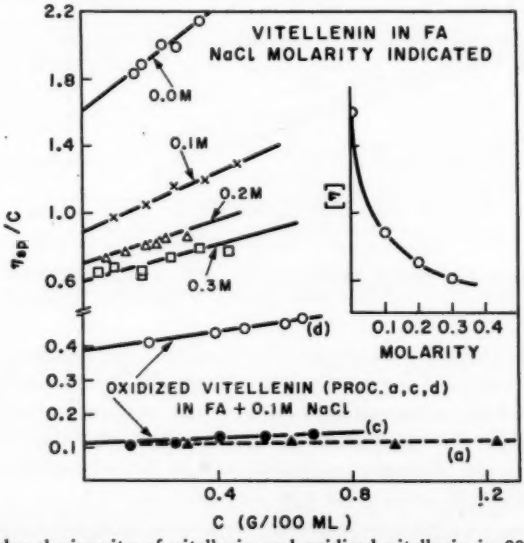

FIG. 4. Reduced viscosity of vitellenin and oxidized vitellenin in 88% w/w formic acid (FA) as a function of concentration. Insert: intrinsic viscosity of vitellenin as a function of salt concentration.

The Archibald procedure, as described for BPA, was used to obtain M_{app} of vitellenin. The relation between M_{app} and c_m was not linear and the method of correcting for concentration dependence, applied to BPA, cannot be considered valid for a polydisperse material (1) since each c_m represents a different spectrum of molecular size. However, concentration dependence was not an important factor in vitellenin solutions and this permitted its polydispersity to be examined by plotting M_{app} versus time (21) instead of versus c_m. In polydisperse solutions the molecules leave the meniscus at differential rates, dependent on the centrifugal force, so that a decrement in both M_{app} and c_m occurs with time. In such systems the plot of M_{app} vs. $t^{\frac{1}{2}}$ is usually linear (21) with a negative slope that differs with the angular velocity. These lines should converge when extrapolated to zero time to yield the mean molecular weight of the original material.

The results for 1:1 : : BPA:oxidized BPA (Fig. 5A) show the negative slope due to polydispersity, but the plots at different concentrations do not coincide and are curved at the higher protein concentrations owing to the dominant

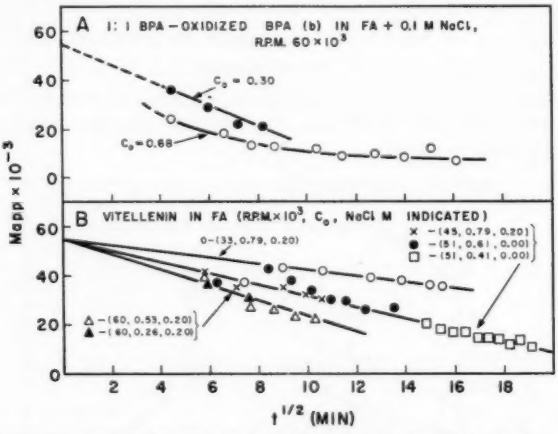

FIG. 5. Apparent molecular weight in 88% w/w formic acid (FA) versus elapsed sedimentation time. (A) 1:1 BPA – oxidized BPA (oxidation procedure (*b*)). (B) Vitellenin.

effect of concentration dependence. In contrast the results with vitellenin (Fig. 5B) in formic acid, with added salt, and at different initial protein concentrations, all fall, within experimental error, on three straight lines whose position and slope are determined by the angular velocities used. While the M_{app} observed under different conditions ranged from 10×10^3 to 43×10^3, the extrapolated plots converge at 55×10^3 at zero time.

The effect on vitellenin of exposure to formic acid was examined by N-terminal amino acid analyses, with the results shown in Table III. Glutamic and aspartic acids were grouped together since they were not adequately

TABLE III

N-Terminal amino acids of vitellenin
(μmoles amino acid/g vitellenin)

Amino acids	Time (days) and temperature in formic acid				
	None	16 at 0° C	76 at 5° C 20 at 20° C	76 at 5° C 36 at 20° C	76 at 5° C 104 at 20° C
Lysine*	3.1	2.9	3.4	2.9	3.1
Alanine Serine Valine	1.3	2.3	2.2	3.4	10.3
Glutamic Aspartic	0.2	0.2	0.5	1.1	0.6

*Lysine corrected for 42% losses reported by Neelin (22).

separated on the chromatograms. The remaining ether-soluble DNP-amino acids were reported as a group since each was present in very minor amounts. The ether-extracted hydrolyzates appeared to contain DNP-arginine but, despite several attempts by paper chromatography, talc column chromatography, electrophoresis, and combinations of these methods (16), it was not possible to obtain quantitative estimates of the apparent DNP-arginine.

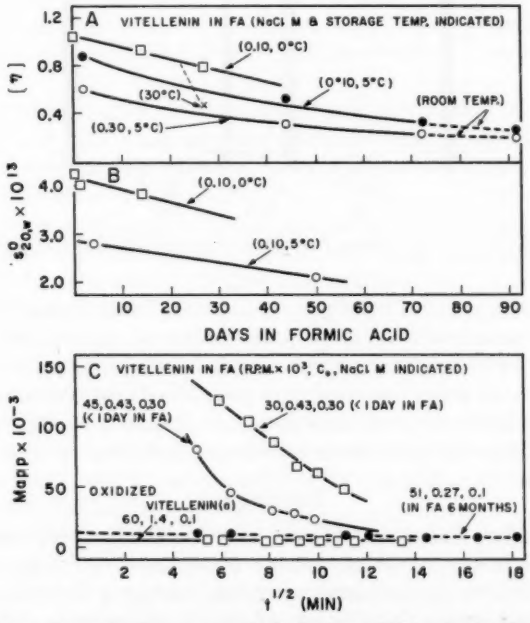

FIG. 6. Effect of length of exposure of vitellenin to 88% w/w formic acid (FA) and change of molecular weight by oxidation. (A) Intrinsic viscosity changes with time and temperature. (B) Sedimentation changes with time and temperature. (C) Apparent molecular weights of vitellenin, after short and lengthy exposure to FA, and of oxidized vitellenin.

Lacking knowledge of the amount of DNP-amino acid in the ether-extracted hydrolyzates, the number average molecular weight could not be estimated with any certainty but, in general, the results were in agreement with those of Neelin (22). Although new N-terminal amino acids were not found on lengthy exposure to formic acid, a slight increase in the total yield of the ether-soluble minor DNP-amino acids occurred after 3 months.

Decreases in $[\eta]$ and sedimentation coefficients of vitellenin occurred with length of exposure to formic acid as shown in Figs. 6A and 6B. The curves labelled 0° C were prepared from measurements on vitellenin, which was dissolved in a tissue grinder cooled by ice water with subsequent storage of the protein solutions at 0° C. As vitellenin was quite difficult to dissolve in 0.3 *M* NaCl – formic acid, these solutions were prepared at room temperature for examination within 24 hours by the Archibald method. Although no apparent aggregates were present, high values of molecular weight were obtained as shown in the plot of M_{app} vs. $t^{\frac{1}{2}}$ in Fig. 6C. Typical schlieren ultracentrifuge patterns of this material are shown in Figs. 7A and 7B.

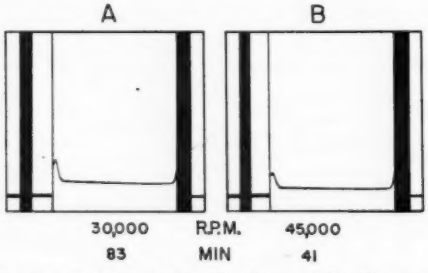

FIG. 7. Schlieren patterns from Archibald ultracentrifugal experiments of vitellenin in formic acid less than 1 day.

A plot of M_{app} vs. $t^{\frac{1}{2}}$ of vitellenin, which had been exposed to formic acid for 6 months, is included in Fig. 6C. The reduction in molecular weight range (13×10^3 to 8×10^3) is quite apparent and is in agreement with the increase in N-terminal amino acids recorded in Table III. However, some degradative oxidation may have occurred since vitellenin, oxidized according to procedure (*a*) (Fig. 6C), was degraded to an even lower molecular weight (6.0×10^3).

Discussion

Omitting values based on viscosity coefficients, because they depend on several assumptions, the more reliable measurements (*s*, *D*, and Archibald procedures) on BPA gave molecular weights averaging 77×10^3 and 70×10^3 in formic acid and formic acid + 0.1 *M* NaCl, respectively. Considering the difficulties imposed by the high density and viscosity of the solvent, this is in reasonable agreement with the probable value of 65×10^3 (23) observed in aqueous solvents, particularly since BPA may contain some higher molecular

weight material. There is therefore no evidence that formic acid degraded BPA into smaller kinetic units.

The mean molecular weight of vitellenin computed from sedimentation and diffusion coefficients was similar to that observed previously (1) when the protein was treated and examined under comparable conditions, but estimates based on sedimentation and viscosity measurements were somewhat higher. Vitellenin dissolved in formic acid at 0° or 5° C and examined within 1 day had higher particle weights (310×10^3) than samples dissolved and held at room temperature for 1 to 2 days before the sedimentation and viscosity measurements were made. Considerably longer periods of exposure to formic acid are required to generate a significant increase in the quantity of N-terminal amino acids in vitellenin or degrade BPA, and it is therefore reasonable to conclude that vitellenin retains its integrity to a comparable degree with that observed for other proteins (2, 3, 4). The decrease in the molecular weight from the values observed initially must therefore be attributed either to the rupture of covalent bonds that do not generate N-terminal amino acids or to the presence of aggregates. The latter appears more probable because the protein moiety of a lipovitellenin fraction of 53% lipid content described by Turner and Cook (24) could only be 160×10^3 in particle weight. Two important inferences are thus raised: formic acid is not a "good" solvent for vitellenin; the presense of aggregates could contribute to the observed polydispersity.

Although vitellenin solutions in formic acid decreased in viscosity and sedimentation rate with time, significant increases in N-terminal amino acids were not detectable within 12-week storage periods. Peptide bond rupture would appear therefore to have little effect within 1 week when most of the present results were obtained. During this period the presence of aggregates would likely have a greater effect with the result that the reported molecular weights are likely to be too high rather than too low.

Formic acid as a protein solvent deserves examination. Because of its unfavorable physical characteristics, accurate measurements are difficult. The 1% increase in $\bar{v}$ of BPA observed in aqueous acid (8) but not in formic acid may be compensated by formylation of serine and threonine. Small primary charge effects (25) in sedimentation experiments in formic acid at less than 0.1 *M* NaCl are in accord with the specific conductivities at 20° C (1.62, 1.78, and 1.64×10^3 cm^{-1} ohm^{-1}, respectively) of formic acid, a dialyzed solution of vitellenin, and its formic acid diffusate.

Although solutions of BPA and vitellenin in formic acid suffered decrements in $[\eta]$ on the addition of salt, the values remained high. As sedimentation experiments revealed no gross aggregates, the decrements, other conditions remaining constant, must be attributed to molecular contraction or electroviscous effects. Consequently, molecular weights based on viscosity measurements and the Scheraga–Mandelkern equation (26) are only useful in showing the gross differences between the unoxidized and oxidized proteins (Tables I

and II) and the changes in vitellenin with dissolution and storage procedures.

The low sedimentation coefficients of BPA in formic acid, compared with those observed in aqueous solution near the isoelectric point, were also noted in acidic aqueous solutions (27, 28) and are probably due to extension or expansion of the macromolecules. The estimated axial ratio of 5 for BPA in acidic solution (27) was assumed to compute the molecular weight of BPA in formic acid from sedimentation and viscosity measurements and the same axial ratio was assumed for vitellenin because it has a similar molecular weight and sedimentation rate.

Contrary to the results in formic acid, performic acid oxidation of BPA decreased the molecular weight of the protein. Reichmann and Colvin (19) reported a less extensive change on oxidation. Later, Jirgensons and Ikenaka (29) observed that some peptide bonds were hydrolyzed by oxidation at room temperature but not at $-7°$ C. Because it was considered desirable to retain the protein in formic acid for physical measurement, performic acid was removed by dialysis versus formic acid at 5° C. Degradation due to residual performic acid during dialysis evidently obscured any differential effects of oxidation at different temperatures. Removal of the performic acid by lyophilization instead of dialysis resulted in a less degraded product but some reaggregation might have occurred.

The position and shape of the curves of the Archibald relations between M_{app} and time depend on the initial protein concentration, degree of concentration dependence, and molecular weight distribution as shown in Fig. 5A for polydisperse 1:1 mixtures of BPA and oxidized BPA. The similar types of curves obtained for vitellenin at several initial concentrations, solvent compositions, and angular velocities provide qualitative evidence of polydispersity. Persistent aggregates may, however, contribute to the polydispersity as high molecular weights were obtained with freshly dissolved vitellenin.

Quantitatively, the molecular weights of vitellenin, omitting conditions in which obvious aggregates or degraded protein existed, ranged from 10×10^3 to 93×10^3 with an extrapolated value of 55×10^3 from Archibald transient state measurements. Because it is difficult to differentiate between natural polydispersity (several N-terminal amino acids were present) and the presence of protein aggregates, the lowest molecular weight may be a good estimate of the minimum size but the maximum size probably exceeds the mean value obtained by sedimentation and diffusion experiments.

Acknowledgments

The author wishes to thank Dr. W. H. Cook for helpful advice and discussions and Mr. J. Giroux for valuable technical assistance.

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STUDIES ON PLASMINOGEN

I. FURTHER PURIFICATION OF BOVINE PLASMINOGEN[1]

EDMOND R. COLE AND EDWIN T. MERTZ

Abstract

Crude bovine plasminogen, prepared from bovine serum by 30% saturation with ammonium sulphate, was partially purified by isoelectric precipitation at pH 5.3, then purified by chromatography on columns of diethylaminoethyl-cellulose. Additional purification of the column product was obtained by precipitation of plasminogen from solution with potassium phosphate. The most highly purified bovine plasminogen preparation, assaying 187 esterase units per mg nitrogen, represents a 28-fold purification of crude plasminogen. The over-all recovery from crude plasminogen was 15–20%. Sodium chloride inhibited crude plasminogen but had no effect on the activation of the most highly purified bovine plasminogen preparations. Bovine plasminogen could be spontaneously activated in 50% glycerol solution, but the degree of activation and the time required for maximum activation was also dependent on the purity of the preparation. Free boundary electrophoresis of purified preparations indicated that bovine plasminogen is associated with the major, faster-migrating component.

Introduction

Plasminogen, the precursor of the proteolytic enzyme plasmin, is a component of blood. Procedures for the purification of human plasminogen have been developed by many investigators (1–5) to the point where sufficient quantities are available for study of its physicochemical properties and physiological role in the fibrinolytic system. Satisfactory methods for the preparation of adequate quantities of bovine plasminogen for use in similar studies have not been reported to date. Bovine plasminogen of relatively high specific activity has been prepared on a small scale on columns of calcium phosphate gel (6) and DEAE-cellulose (7).

A study of the optimum conditions for chromatography of bovine plasminogen on DEAE-cellulose columns has resulted in the development of a fast and reproducible chromatographic method for the purification of bovine plasminogen. Further purification of these column preparations was achieved by taking advantage of the precipitation of plasminogen in the presence of phosphate buffer, under special conditions.

Experimental

Bovine Plasminogen

Bovine plasma[2] was used as the source of plasminogen. The oxalated plasma was received frozen in 50-pound cans and allowed to thaw in a 5° cold room.

[1]Manuscript received April 21, 1961.
Contribution from the Department of Biochemistry, Purdue University, Lafayette, Indiana. Journal Paper No. 1744, Purdue University Agricultural Experiment Station. Supported by Contract DA-49-193-MD-2091, Office of the Surgeon General, Department of the Army.

[2]Swift and Company.

The plasma was defibrinated and brought to 30% saturation with ammonium sulphate according to the procedure of Jackson and Mertz (8), and the precipitate was collected by centrifugation and subjected to isoelectric precipitation at pH 5.3 and 40-fold dilution, by methods previously reported (6). The precipitate was dissolved in water together with sufficient 0.1 *M* Na_2HPO_4 to give a final concentration of 0.01 *M* Na_2HPO_4 and a volume of 1/25th the original serum volume. Before making up to volume, the pH was adjusted to 8.9 with 0.1 *N* NaOH. The plasminogen solution was stored frozen and designated as bovine IEP preparation. Three IEP preparations were used as the starting material for DEAE-cellulose column chromatography and had specific activities of 2.5, 2.0, and 2.6 esterase units (9) per unit optical density for IEP-1, IEP-2, and IEP-3 preparations, respectively. Another plasminogen preparation, IEP-4, was lyophilized after isoelectric precipitation and adjustment of the pH to 7.4 with NaOH. The lyophilized IEP-4, assaying 2.0 esterase units per unit optical density, was used in other experiments described in this report.

Human Plasminogen

Human plasminogen was partially purified from fraction III[3] by the method of Kline (3). The preparation used in these experiments had a specific activity of 5.9 esterase units per unit optical density.

Plasmin Assays

The Warburg manometric technique adapted for the assay of plasmin by Dalby and Mertz (9) was used and is based on the esterase activity which plasmin shows towards *p*-toluenesulphonyl-L-arginine methyl ester (TAMe). Bovine and human plasminogen preparations were activated with 1% SKHG (streptokinase[4]-human globulin) at 25° for 1 hour in the presence of 0.01 *M* potassium phosphate buffer, pH 7.4 (9). The plasminogen concentration of the activation mixture was adjusted so that 0.2 ml of activation mixture yielded 3–6 esterase units in the assay system. Plasmin activity was corrected for the small amount of esterase activity due to the SKHG activator.

Proteolytic activity of plasminogen preparations was determined by measurement of the hydrolysis of casein as described by Remmert and Cohen (1). Both bovine and human plasminogen were activated by 0.2 ml of SKHG (9), an amount which was found sufficient for complete activation of plasminogen in 10 minutes. Proteolytic activity was corrected for SKHG activity.

Determination of Protein Concentration

Specific activities of plasminogen preparations are given in terms of the yield of plasmin and expressed as esterase units or casein units per unit of optical density. The optical density of the preparations, dissolved in 0.1 *N* NaOH, was measured at 280 mμ in a Beckman DU spectrophotometer. In some cases, protein concentration was also determined by a micro-Kjeldahl technique, and specific activities are expressed as esterase units or casein units per milligram

[3]Fraction III was obtained through the courtesy of E. R. Squibb and Sons and Dr. J. H. Pert of the American Red Cross.

[4]Varidase, Lederle Laboratories.

of nitrogen. Based on these observations, 1 mg protein nitrogen is approximately equal to 10 units of optical density.

Chromatographic Columns

Pyrex glass columns, 2.5 cm in diameter and 60 cm in length, were used. A pad of glass wool was laid in the bottom of the column and covered with 2 cm of sand. Twenty-five grams of 200–325 mesh DEAE-cellulose[5] was weighed out in a beaker, a thick aqueous slurry was prepared, and 200 ml of 1 *N* NaOH was mixed in. The suspension was filtered on a sintered glass filter of coarse porosity to remove the yellow color found in the commercial preparations of DEAE-cellulose, and the DEAE-cellulose was washed four times with 200 ml of water and filtered, and the cake dried to the cracking point with gentle suction between washings. The DEAE-cellulose was suspended in 250 ml of 0.01 *M* sodium phosphate buffer, pH 7.4, and slowly added to the column, allowing the DEAE-cellulose to fall through a column of buffer. By leaving the outlet end of the column open, an evenly packed column could be obtained. Two hundred milliliters of 1 *N* NaOH was passed through the column to compress the column to a height of 48–50 cm, and the column was equilibrated with 0.01 *M* sodium phosphate buffer, pH 7.4, by allowing approximately 1 liter of buffer to run through the column overnight. The conductivity of the eluate became constant within 3 hours after adding 0.01 *M* phosphate buffer to the column.

Chromatographic Procedure

Ten to twelve milliliters of bovine IEP, representing 83.5–100 mg of nitrogen and 2050–2180 esterase units, was layered under the 0.01 *M* sodium phosphate buffer, pH 7.4, at the top of the column and allowed to run into the column. This was followed by buffer in the following concentrations: 100 ml of 0.01 *M*, 150 ml of 0.03 *M*, and approximately 350 ml of 0.05 *M*. Material eluted by the 0.01 *M* and 0.03 *M* buffers was discarded, but as the molarity of the eluate buffer increased beyond 0.03 *M*, as measured by a flow-through conductivity cell (10), collection of the eluate was started. The eluate was collected in a 400-ml beaker in an ice bath. All column experiments were run at room temperature and at a flow rate of about 100 ml/hour, without the use of pressure. Two hundred milliliters of the eluate was collected and dialyzed against 4 liters of ice cold 0.02 *N* HCl for 1 hour. The material was then dialyzed in a 5° cold room for 18–20 hours against two changes of distilled water, using a dialysan: dialysate ratio of 1:40. The material was then removed from the dialysis bag, adjusted to pH 7.4 with 0.1 *N* NaOH, and lyophilized. The lyophilized material was stored at −10° C in stoppered glass vials.

The procedure described above was used for routine purification of bovine plasminogen. In preliminary experiments, the procedure was varied in order to determine the optimum chromatographic conditions, as indicated in the Results section.

[5]Selectacel-DEAE, Reagent Grade, Brown Company, Berlin, N. H.

Potassium Phosphate Precipitation of Bovine Plasminogen

Lyophilized bovine plasminogen preparations from DEAE-cellulose columns were soluble in distilled water, and the addition of potassium phosphate buffer to these solutions resulted in a selective precipitation of plasminogen.

Several lyophilized plasminogen preparations from DEAE-cellulose columns were pooled and dissolved in water to obtain a final concentration of 5 mg/ml, after the addition of potassium phosphate buffer. In most cases, dissolution was aided by stirring. After dissolution of the protein, potassium phosphate buffer (0.1 *M*, pH 7.4) was added slowly to yield a final concentration of 0.02 *M* phosphate. A precipitate formed instantly and stirring was continued for 15 minutes at room temperature. The suspension was then set in an ice water bath for 15 minutes, with occasional stirring with a glass stirring rod. The suspension was centrifuged for 15 minutes, and the supernatant was discarded. The precipitate was taken up in distilled water to yield a protein concentration of about 70 to 80 mg/100 ml and dialyzed against ice-cold 0.02 *N* HCl for 1 hour. Dialysis against distilled water followed under the conditions described for DEAE-cellulose column fractions. The dialyzed solution was adjusted to pH 7.4 with 0.1 *N* NaOH and lyophilized. Reprecipitation of plasminogen could be carried out by following the procedure above. These lyophilized plasminogen preparations were stored at $-10°$ C in stoppered glass vials. In preliminary experiments, conditions for precipitation sometimes varied from those described above, as indicated in the Results section.

Free Boundary Electrophoresis

Electrophoretic studies were made in a Perkin–Elmer Model 38 instrument, using standard 2-ml cells. Glycine–HCl (pH 2.5) and acetate (pH 4.0) buffers were used, each with an ionic strength of 0.1. Protein concentrations were approximately 1% in all samples. The temperature was maintained at 0° C, and conductivity measurements were also made at this temperature. Because of the limited amounts of purified plasminogen preparations available for electrophoresis, conductivity measurements were made on the buffer against which the protein solution was dialyzed, rather than on the dialyzed protein solution.

Starch Gel Electrophoresis

The method of starch gel electrophoresis of purified bovine and human plasminogen preparations was that of Smithies (11), except that glycine–HCl buffer, pH 2.1 and 0.1 ionic strength, was used in place of borate buffer. Amend[6] potato starch was hydrolyzed for 43 minutes with an acetone–HCl mixture, washed, and dried according to the method of Smithies (11). The gel was prepared in the usual way by mixing 15 g of the hydrolyzed starch with 100 ml of glycine–HCl buffer, heating and pouring the hot gel into plastic electrophoresis trays (295×20×6.5 mm). The plasminogen samples were prepared by dissolving 15 mg of lyophilized preparation in 1 ml of pH 2.1 glycine–

[6]Amend Drug and Chemical Company, Inc.

HCl buffer and adding unhydrolyzed starch to make a thick paste which still could be drawn into a 1-ml serological pipette. A 2.5-mm slot cut out of the gel about half-way down the length of the gel tray was filled with the paste. Electrophoresis was carried out at 3 volts/cm for 16 hours in a 5° cold room.

Results

DEAE-cellulose Column Chromatography

1. Purification by the Standard Method

Data obtained from the use of the standard method (see Experimental section) on three IEP preparations are shown in Table I. In order to chromatograph approximately the same number of plasminogen units, 10 ml of IEP-1

TABLE I

DEAE-cellulose column chromatography of bovine plasminogen

Preparation chromatographed	Lyophilized product				
	mg	Esterase, units/mg	Specific activity*	Recovery of activity, %	Purification
IEP-1†	145 (120–162)	7.9 (6.5–9.9)	6.8 (5.5–8.8)	53 (40–60)	2.7-fold (2.2–3.5)
IEP-2‡	144 (105–196)	7.0 (5.0–8.2)	6.3 (5.4–7.2)	49 (38–68)	3.1-fold (2.6–3.6)
IEP-3§	187 (137–224)	7.5 (7.2–7.8)	8.1 (7.5–8.9)	66 (50–78)	3.1-fold (2.9–3.4)

*Esterase units/unit optical density.
†Data represents 5 chromatographic runs. Specific activity of IEP-1: 2.5 esterase units/unit optical density.
‡Data represents 18 chromatographic runs. Specific activity of IEP-2: 2.0 esterase units/unit optical density.
§Data represents 10 chromatographic runs. Specific activity of IEP-3: 2.6 esterase units/unit optical density.

(2180 units, 86.5 mg N) and IEP-3 (2130 units, 83.5 mg N) or 12 ml of IEP-2 (2050 units, 100 mg N) were used. The specific activity and recovery of plasminogen in the lyophilized column eluate appears to be dependent on the specific activity of the IEP preparation chromatographed.

2. Effect of Variations in the Method on Elution Patterns

Shown in Fig. 1 is an elution diagram of bovine plasminogen chromatographed on DEAE-cellulose. This diagram was obtained by the elution of 10 ml of IEP-1 from an equilibrated 20-g column of DEAE-cellulose with 100 ml of 0.01 *M*, 100 ml of 0.03 *M*, and 200 ml each of 0.05 *M* and 0.1 *M* sodium phosphate buffer, pH 7.4. Also shown is the distribution of plasminogen among the various peaks. Most of the plasminogen was eluted by 0.05 *M* buffer in fractions 32 to 40. The preparative chromatographic procedure described in the Experimental section was developed and based on the data obtained by varying the elution conditions during preliminary experiments. Phosphate buffer ranges wider than 0.03–0.05 *M* tended to increase the amount of plasminogen eluted from the column, but the specific activity of these preparations was lower. Conversely, narrower phosphate buffer ranges resulted in higher

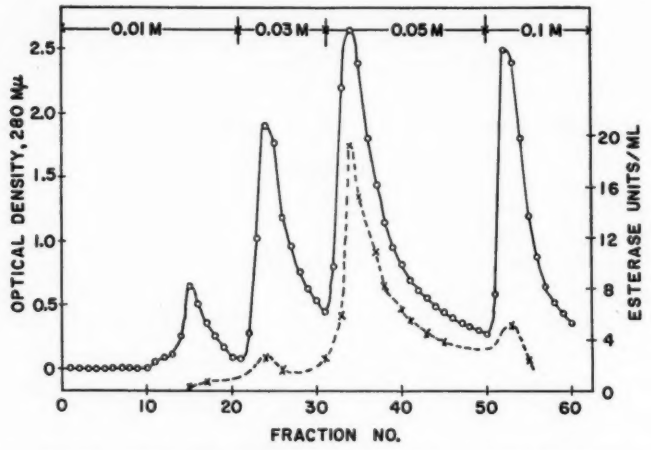

FIG. 1. Stepwise elution of bovine plasminogen from DEAE-cellulose columns. (○ optical density; × esterase units/ml).

specific activity of the preparations, but the yield was too low for routine purification of plasminogen. Slightly higher specific activity of plasminogen was obtained when 150 ml of 0.03 *M* buffer preceded elution with 0.05 *M*. Twenty-five-gram DEAE-cellulose columns yielded plasminogen of higher activity than 20-g columns.

After use, DEAE-cellulose columns could be cleared of remaining protein by elution with 250 ml of 1 *N* NaOH and re-equilibrated with 0.01 *M* sodium phosphate buffer, pH 7.4. This alkali treatment had no significant effect on the chromatographic properties of DEAE-cellulose, and DEAE-cellulose columns could be used repeatedly for chromatography of bovine plasminogen. However, a reduction in eluate flow rate was generally observed after three runs, and the columns were, therefore, repacked at the end of this time.

Although the columns were equilibrated with 0.01 *M* sodium phosphate buffer, pH 7.4, to constant conductivity of the eluate, the pH of the eluate from the DEAE-cellulose columns was approximately 10.4. This pH was found to be desirable. Whenever columns were prepared so that the eluate pH was near 7.4, the yield and specific activity of the plasminogen obtained was much lower than that obtained with the standard method.

Potassium Phosphate Precipitation of Bovine Plasminogen

Prior to the assay of DEAE-cellulose column preparations of plasminogen for esterase activity, the lyophilized material was dissolved in water and sufficient 0.1 *M* potassium phosphate buffer, pH 7.4, was added to bring the phosphate molarity to 0.01. It was observed that whereas the plasminogen preparation was soluble in distilled water, the addition of the phosphate buffer caused an immediate formation of precipitate. After addition of SKHG acti-

vator, the precipitate slowly disappeared, and, at the end of the 60-minute activation period, only traces of precipitate remained. This precipitation reaction was investigated as a means of bovine plasminogen purification, and it was found that bovine plasminogen was precipitated in the presence of potassium phosphate buffer.

The data obtained from five experiments in which chromatographed bovine plasminogen was precipitated with potassium phosphate according to the standard method (see Experimental section) are shown in Table II. Several

TABLE II

Potassium phosphate precipitation of bovine plasminogen

	Pooled column fractions			Lyophilized precipitate		
Experiment	Specific activity*	mg	Phosphate molarity	Specific activity*	mg	Recovery of plasminogen, %
1	6.8	220	0.01	10.6	76	65
2	5.8	710	0.02	8.9	220	59
3	6.2	540	0.02	9.2	186	61
	9.2†	156	0.02	15.3	85	99
4	6.4	1422	0.02	13.9	382	71
	13.9‡	372	0.02	16.9	195	68
5	7.6	1450	0.02	14.0	356	64

*Esterase units/unit optical density.
†Second phosphate precipitation of plasminogen obtained in experiment 3.
‡Second phosphate precipitation of plasminogen obtained in experiment 4.

lyophilized plasminogen preparations from DEAE-cellulose columns were pooled to obtain sufficient quantities of material for these experiments. Experiments 1, 2, and 5 represent a single precipitation of plasminogen from column preparations. In experiments 3 and 4, however, a second precipitation was performed on dialyzed and lyophilized material obtained by phosphate precipitation of pooled DEAE-cellulose column preparations. Bovine plasminogen assaying 16.9 esterase units per unit optical density represents the maximum purification that has been achieved by these preparative techniques. The amount of plasminogen that can be recovered by phosphate precipitation is variable. The over-all recovery of plasminogen in experiments 3 and 4 was 60% and 48%, respectively. Recovery of plasminogen in the second precipitation step of experiment 3 was nearly quantitative compared with a 68% recovery in experiment 4. Additional studies are needed to explain these differences.

In Table III, the effect of variations in precipitation conditions are summarized. These data were obtained from 10-mg samples of plasminogen preparations which were dissolved in water to obtain a final concentration of 5 mg/ml, after the addition of potassium phosphate buffer. After dissolution of the sample, the phosphate buffer (0.1 *M*, pH 7.4) was added to give the desired final molarity of phosphate, and the suspension was incubated 5 minutes at room temperature and 5 minutes in an ice bath. The precipitates were

TABLE III

Summary of data from preliminary experiments on potassium phosphate precipitation of bovine plasminogen

Experiment	Specific activity of starting material*	Final phosphate molarity	Specific activity of precipitate*	Plasminogen recovery, %
1	6.6	0.01	11.0	66
2	4.9	0.01	9.5	87
3	8.8	0.01	11.5	83
4	10.6	0.01	17.6	64
5	2.0	0.01	2.2	20
		0.02	3.1	48
		0.03	2.7	30
		0.04	2.1	22
		0.05	0.6	8
6	4.1	0.01	3.9	66
		0.02	5.0	76
		0.04	4.9	70
7	5.6	0.01	9.5	61
		0.02	10.8	89
		0.03	10.0	83
8	9.2	0.01	15.0	84
		0.02	14.8	94

*Esterase units/unit optical density.

centrifuged out, the supernatant was discarded, and the precipitates were suspended in distilled water and assayed. In general (Table III), the specific activity of the precipitate is dependent on the specific activity of the starting plasminogen preparation, and upon the final phosphate concentration. From these data, it appears that significant purification cannot be achieved by phosphate precipitation with preparations assaying less than about 4.9 esterase units per unit optical density. Data from experiments in which the final phosphate concentration was varied (Table III, experiments 5, 6, and 7) have shown that maximum purification and yield of plasminogen is achieved with a final phosphate molarity of 0.02.

Effect of Chloride Ion on Activation of Bovine Plasminogen

Dalby and Mertz (9) observed that sodium chloride inhibits the activation of crude bovine plasminogen by streptokinase and human globulin activator. In the current study, chloride inhibition of bovine plasminogen activation at a 1% SKHG level was studied using plasminogen preparations of varying specific activities. The degree of inhibition was found to be dependent on the purity of the plasminogen. Phosphate-precipitated bovine plasminogen and human plasminogen showed no chloride inhibition to activation, but plasminogen preparations of lower purity, namely, 30% ammonium sulphate precipitate from serum, and bovine IEP, were susceptible to chloride inhibition up to a 0.06 *M* NaCl level. Concentrations of chloride higher than this were not studied.

Glycerol Activation of Bovine Plasminogen

Alkjaersig *et al.* (12) were the first to show that human plasminogen can be spontaneously activated in a 50% glycerol solution. Bovine plasminogen preparations of varying specific activity have been incubated at 25° in a 50% glycerol – 0.05 *M* sodium phosphate buffer, pH 7.4. The plasminogen content of the activation mixtures was 30–33 esterase units/ml. A bovine plasminogen preparation, prepared by phosphate precipitation and assaying 16.9 esterase units per unit optical density, attained a maximum activation of 85% in 5 days. A DEAE-cellulose column preparation, assaying 7.5 esterase units per unit optical density, reached maximum activation of 60% in 11 days. Bovine IEP-4, 2.0 esterase units per unit optical density, reached 45% activation in 22 days. The plasmin formed at 25° appeared to be stable for several days. Another DEAE-cellulose column preparation, assaying 8.9 esterase units per unit optical density, was activated in 50% glycerol at 37° and attained a maximum activation of 55% in 2 days, but this was decreased to 45% by the fourth day, indicating that the bovine plasmin was not as stable at this higher temperature. These data show that the degree and rate of activation depended on the purity of the bovine plasminogen.

Free Boundary Electrophoresis

Plasminogen preparations obtained at each stage of the purification method were subjected to free boundary electrophoresis, and these patterns are illustrated in Fig. 2. At pH 2.5, a slow-migrating boundary was observed, and the size of this boundary did not change significantly in electrophoretic patterns of plasminogen preparations of increasing specific activity. This boundary was not observed at pH 4.0. As the specific activity of the plasminogen preparations increased, electrophoretic patterns showed that the size of the fastest-migrating boundary increased, and this was taken as evidence that the major, fastest-migrating boundary represented or included the plasminogen boundary. At pH 2.5, the preparation assaying 16.9 esterase units per unit optical density (IV) appeared to contain at least two minor components which migrate at a slower rate than the major boundary. The number of components at pH 4.0 (V) was more difficult to detect, but the dissymmetry of the boundary suggested that at least one minor component was present. Mobilities of the major boundaries of bovine and human plasminogen, calculated from the descending patterns, were 7.5 and 3.8×10^{-5} cm^2 per volt per second for bovine plasminogen at pH 2.5 and pH 4.0, respectively, while the mobility of human plasminogen was 7.8×10^{-5} cm^2 per volt per second at pH 2.5.

To determine if the faster-migrating, major boundary was bovine plasminogen, a DEAE-cellulose column preparation of bovine plasminogen assaying 6.6 esterase units per unit optical density was activated with SKHG at a 1% level in the presence of 0.01 *M* potassium phosphate, pH 7.4, at a temperature at 25°. At the end of the activation period, the mixture was dialyzed at 5° for at least 24 hours against three 500-ml changes of glycine–HCl buffer, pH 2.5.

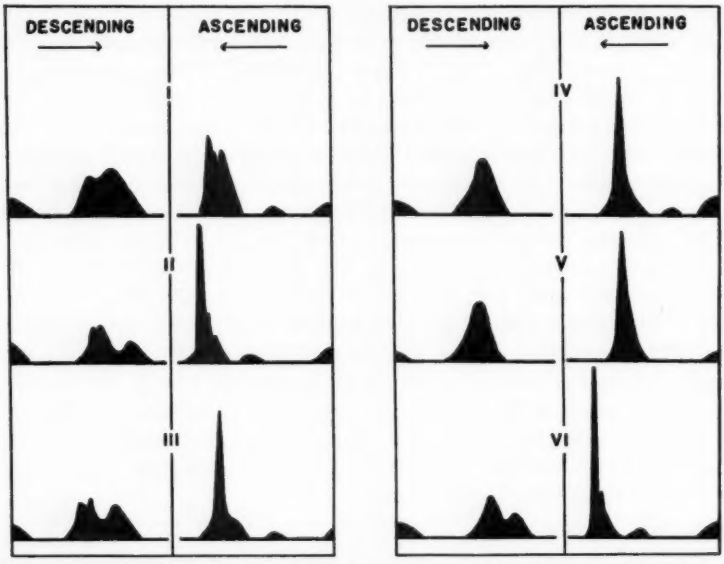

FIG. 2. Electrophoretic patterns of bovine and human plasminogen preparations. All protein concentrations were near 1%.
(I) Bovine IEP, 2.5 esterase units/unit O.D. (pH 2.5, 7.2 volts/cm, 105 minutes); (II) DEAE-cellulose column preparation, 6.6 esterase units/unit O.D. (pH 2.5, 7.2 volts/cm, 105 minutes); (III) phosphate precipitated bovine plasminogen, 10.6 esterase units/unit O.D. (pH 2.5, 6.7 volts/cm, 105 minutes); (IV) phosphate precipitated bovine plasminogen, 16.9 esterase units/unit O.D. (pH 2.5, 6.8 volts/cm, 78 minutes); (V) same as IV except run at pH 4.0, 11.0 volts/cm, 90 minutes; (VI) human plasminogen, 5.9 esterase units/unit O.D. (pH 2.5, 7.0 volts/cm, 105 minutes).

Electrophoretic analysis of the preparation before activation and after 1 and 2 hours' activation was made. These electrophoretic patterns are illustrated in Fig. 3, which shows the disappearance of the faster-migrating, major component. However, the appearance of a plasmin boundary has not been demonstrated, even though assays of the activation mixture after dialysis and after electrophoresis have shown that plasmin esterase activity is present.

Starch Gel Electrophoresis

A photograph of the starch gel strips used for the electrophoresis of bovine plasminogen (16.9 esterase units per unit optical density) and human plasminogen (5.9 esterase units per unit optical density) is shown in Fig. 4. Both preparations were run at the same time and at the same electrical potential. Four bands were detected in the stained gel strips on which bovine plasminogen was subjected to electrophoresis. One trace component migrated in front of the major component and has not been detected in free boundary electrophoresis. The major component and the two slower-migrating components formed a pattern similar to that observed in free boundary electrophoresis. It is interesting to note that the fastest-migrating trace component of bovine plasminogen

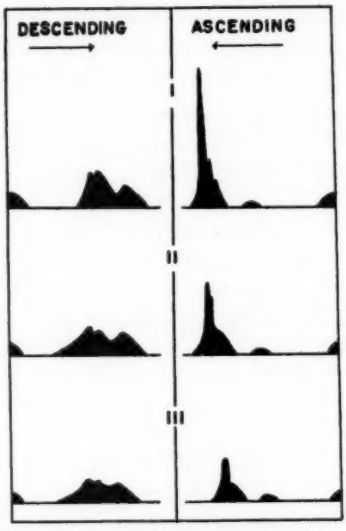

FIG. 3. Electrophoretic patterns of bovine plasminogen before and after activation with 1% SKHG. Specific activity of the preparation: 6.6 esterase units/unit O.D. Initial concentration of protein: 1.0%. Glycine–HCl buffer (pH 2.5, ionic strength 0.1, 7.2 volts/cm, 105 minutes). (I) Before activation; (II) after 1 hour activation; (III) after 2 hours' activation.

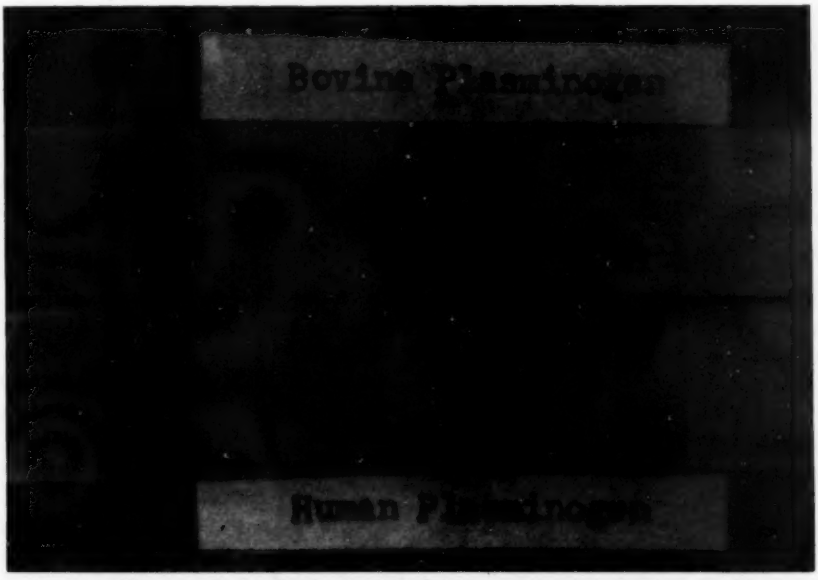

FIG. 4. Photograph of stained gel strips used for starch gel electrophoresis of bovine and human plasminogen. Migration is to the left.

has the same migration rate as the major component of human plasminogen, while the major component of bovine plasminogen has the same migration rate as the second-fastest-migrating component of human plasminogen.

Proteolytic Activity of Bovine and Human Plasminogen

The specific activity of bovine plasminogen preparations, obtained at various stages of purification and activated by SKHG, has also been followed by a casein hydrolysis method (1). The most active bovine plasminogen preparation obtained had a specific activity of 16.9 esterase units per unit optical density and 2.1 casein units per unit optical density. Based on the specific activity of the 30% ammonium sulphate precipitate from bovine serum, this was a 28-fold purification by esterase assay and a 26-fold purification by casein assay. The human plasminogen preparation assayed 5.9 esterase units per unit optical density and 2.0 casein units per unit optical density. On a nitrogen basis, the specific activity of the bovine plasminogen preparation was 187 esterase units per mg N and 23 casein units per mg N, and the specific activity of the human plasminogen preparation was 60 esterase units per mg N and 20 casein units per mg N.

Discussion

Chromatography of bovine plasminogen on columns of DEAE-cellulose has proved efficient in removing those impurities which prevent precipitation of plasminogen by potassium phosphate. There appears to be a limiting level of purity below which potassium phosphate precipitation is not effective. It would have been desirable for bovine IEP preparations to have been sensitive to the potassium phosphate precipitation method, since relatively large amounts of plasminogen could then be purified by simple techniques on a batchwise scale. However, the purity of IEP preparation was not sufficiently high, and the DEAE-cellulose column chromatography procedure was necessary. Elution of plasminogen in buffer ranges narrower than 0.03–0.05 *M* tended to increase the specific activity of the plasminogen preparations, but a decrease in recovery of plasminogen from the column accompanied this increase in specific activity. Therefore, the preparative method of DEAE-cellulose column chromatography was designed not to yield plasminogen of the highest possible purity, but to yield plasminogen of sufficient purity to allow further purification by the potassium phosphate purification method.

Certain observations concerning the phosphate precipitation method should be mentioned. In almost all cases, the plasminogen preparations purified with DEAE-cellulose columns were soluble in distilled water. Occasionally, a preparation would be obtained which lacked this complete solubility. In such cases, the preparation was suspended in the water, potassium phosphate was added, and the purification obtained was equal to that obtained with soluble preparations of similar purity. The only failures to achieve purification of plasminogen by this method have come from dialyzing the precipitate at a high concentration of protein. In these cases, dialysis against water to remove the

HCl and potassium phosphate resulted in protein precipitation and the preparation was no longer active. To prevent this, precipitates obtained with potassium phosphate were suspended in water to a concentration of 70–80 mg protein/100 ml, and on subsequent dialysis against HCl and water, no precipitation occurred. Under these conditions, no difficulty has been encountered in obtaining highly purified preparations.

The nature of the precipitation by potassium phosphate is not known. Kline (3) employed this technique in purifying human plasminogen, but the conditions and buffer concentrations were different from those employed here.

About the same degree of purification is calculated whether the purification is based on TAMe esterase units or casein units. Data are not available to calculate purification from serum since bovine serum contains large amounts of antiplasmin, and attempts to obtain meaningful plasminogen values in the presence of this inhibitor have been unsuccessful. However, on the assumption that a quantitative recovery of plasminogen from serum is achieved by ammonium sulphate precipitation, a maximum purification of 160- to 170-fold can be calculated for the most active bovine plasminogen preparations obtained. Because of the dependence on the specific activity of the starting material in each stage of purification and other variables which affect the over-all recovery of plasminogen, it is difficult to calculate percentage yield. However, using average values, an over-all yield of 15 to 20% is calculated for the most active bovine plasminogen preparations obtained, starting with the 30% ammonium sulphate precipitate (crude plasminogen) from serum.

It is interesting to note that the ratio of the esterase activity to caseinolytic activity is about 8 for the most active bovine plasminogen preparation (187 esterase units per mg N/23 casein units per mg N), while the ratio is about 3 for the human plasminogen preparation (60 esterase units per mg N/20 casein units per mg N). This difference in activity toward the two substrates has been studied further and will be the subject of a later communication.

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EFFECT OF ANTIBODIES ON REMOVAL OF INSULIN FROM THE CIRCULATION[1]

NORMAN KALANT, REUBEN SCHUCHER, AND WILLIAM C. GALLEY

Abstract

Guinea pigs have been immunized against insulin by the injection of insulin in Freund's adjuvant. All animals developed high titers of antibodies and a decreased hypoglycemic response to insulin; some showed delayed removal of a test dose of insulin from the circulation, while others showed a normal removal rate. These effects were also found in animals passively immunized by the infusion of sera from actively immunized animals. The differences in the rate of removal of insulin from serum in immunized animals may be due to differences in the removal of the insulin–antibody complex.

Evidence concerning the antigenicity of insulin has been recently reviewed by Wright (1). Antibodies to insulin may be associated with a decrease in the rate at which insulin is removed from the circulation (2, 3), and the suggestion has been made, not unreasonably, that binding of insulin to antibodies is in fact responsible for the decreased removal rate (2). The latter concept was questioned when it was shown in rabbits passively immunized by the administration of human anti-insulin serum that the presence of antibodies was *not* associated with slow removal of insulin from the circulation. On the basis of this evidence an alternative hypothesis was presented to explain the relationship between antibodies and "plasma retention" of insulin (4). However, the possibility existed that these results were attributable to the use of heterologous antiserum and to differences in the removal rates from the circulation of the antigen–antibody complexes. Further studies have now been carried out to clarify this point.

Methods

(*a*) Guinea pigs were immunized by the administration of insulin in Freund's adjuvant. Crystalline insulin was dissolved in 0.9% NaCl and the pH adjusted to 2.0 with HCl; this was emulsified with adjuvant (complete adjuvant, Difco Laboratories) manually by repeated ejection from a syringe and needle, or in a high-speed homogenizer. An initial injection of 0.25 mg insulin in 1 ml of emulsion containing 0.5 ml adjuvant was given subcutaneously over the adbomen. One month later, 0.25 mg was given in 0.5 ml of emulsion containing 0.25 ml of adjuvant.

(*b*) The rate of insulin disappearance from the circulation was measured as previously described (4) with plasma samples being taken at intervals up to 45 minutes following the intravenous injection of I^{131}-insulin (0.4 unit/kg).

[1]Manuscript received May 5, 1961.
Contribution from the Research Laboratory, Jewish General Hospital, and the Department of Investigative Medicine, McGill University, Montreal, Que. Supported by a grant from the National Research Council of Canada.

A control animal was tested simultaneously with each immunized animal.

(*c*) Insulin responsiveness was measured as the response to intravenously administered crystalline insulin (0.2 to 0.75 unit/kg body weight); blood sugar concentrations, 5, 10, 15, 20, 30, 40, and 50 minutes after insulin, were determined by the Somogyi (5) modification of the Nelson method. The values were plotted against time and the area enclosed by the curve below a base line, drawn at the level of the initial concentration, was measured by planimetry. In procedures (*b*) and (*c*) animals were anesthetized with sodium pentobarbital and a fine polyethylene catheter was inserted into an external jugular vein. The catheter was filled with heparin solution (100 units/ml) and clamped after each blood sample was withdrawn. At the time of blood collection, the first eight to nine drops of blood issuing from the catheter were discarded.

(*d*) Plasma volume was measured by the use of I^{131} human serum albumin (6), at a dose of 1.0 mg containing 25 μc dissolved in 0.25 ml of saline. To 200 μl of plasma were added 1 ml of water and 1 ml of 20% TCA. The precipitate was washed twice with 20% TCA, dissolved in 10% NaOH, and

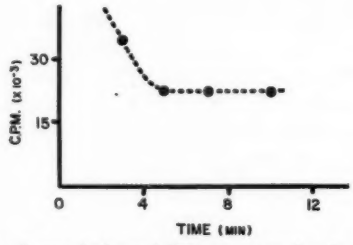


FIG. 1. Time course of protein-bound I^{131} concentration in guinea pig plasma after injection of I^{131}-albumin. Counts per minute per ml of plasma against time.

counted in a well scintillation detector. In a preliminary experiment a plateau in serum protein radioactivity was found between 5 and 9 minutes after albumin-I^{131} administration (Fig. 1). In subsequent measurements, single samples were taken at 6 minutes. In a group of five animals there was found to be a close relationship between plasma volume and body weight (Fig. 2).

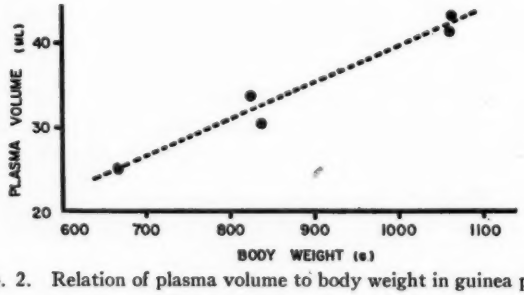


FIG. 2. Relation of plasma volume to body weight in guinea pigs.

(*e*) Titers of insulin antibody in serum were determined in vitro employing procedures described by Berson and Yalow (2). Insulin-I^{131} was obtained from Abbott Laboratories. Less than 5% of the radioactivity behaved differently from insulin on chromatography and was considered to have been altered by radiation (7). Estimates were made of the maximum amount of insulin which could be completely bound by each serum.

Results

(*a*) *Relation of Antibodies to Rate of Insulin Disappearance*

Two groups of guinea pigs were immunized with insulin, the second group about 3 months after the first. In each case the titer of antibodies and the rate of I^{131}-insulin disappearance were measured. In Table I are shown the

TABLE I

Relation of binding capacity to dose of insulin in actively immunized guinea pigs

Source of antiserum	Insulin completely bound in vitro (μg/ml)	Plasma volume (ml)	In vivo binding ability (μg/P.V.)	Insulin administered for disappearance test (μg)
Group 1	> 40	30 ± 3	> 1200 ± 100	< 15
Group 2	> 15	29 ± 2	> 435 ± 30	< 15

insulin binding capacities, the maximum amounts of insulin completely bound in vivo (determined from the binding capacity and the plasma volume), and the amounts of insulin given in the disappearance tests. It is seen that in each case the test dose could be completely bound. It is apparent, however (Fig. 3),

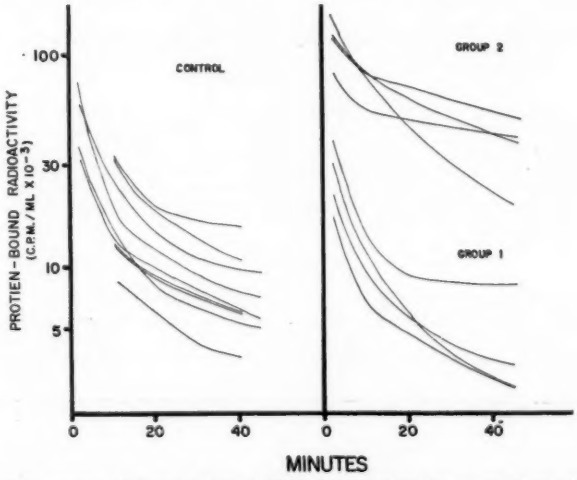


FIG. 3. Time course of protein-bound I^{131} concentration in serum after administration of I^{131}-insulin. Groups 1 and 2 are animals immunized against insulin.

that in group 1 the disappearance of insulin was unimpaired while in group 2 it was markedly delayed.

(*b*) *Effects of Passive Immunization on Insulin Disappearance*

Sera from one animal of group 1 (the remainder having died) and from all four animals of group 2 were used to immunize normal guinea pigs. The recipient animals were anesthetized and given 5 ml of antiserum, over a

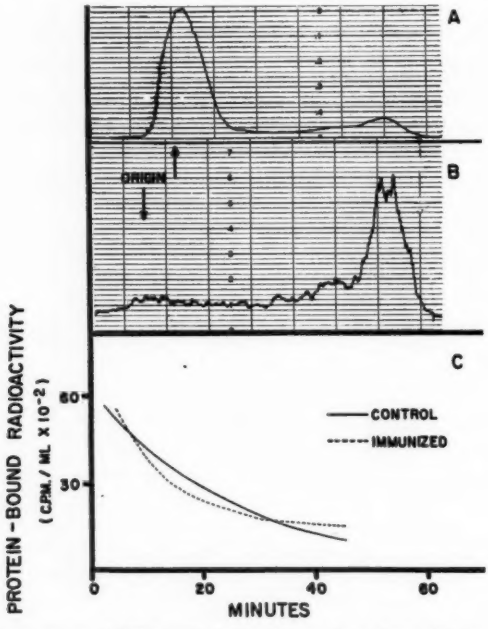


FIG. 4. Disappearance of I^{131}-insulin from plasma in guinea pig passively immunized with anti-insulin serum from animal in group 1. Distribution of radioactivity after chromatography of serum, 2 minutes after administration of I^{131}-insulin to control animal (A) and to passively immunized animal (B). Time course of protein-bound I^{131} concentration in plasma (C). Insulin bound to antibodies moves forward on the chromatogram; insulin that is "free" in the serum is absorbed on the filter paper and remains at the origin during chromatography.

TABLE II

Relation of binding capacity to dose of insulin in passively immunized guinea pigs

Source of antiserum	Insulin completely bound in vivo (μg)	Insulin administered for disappearance test (μg)
Group 1	> 44	10
Group 2	14	12
	9	12
	48	11
	32	12

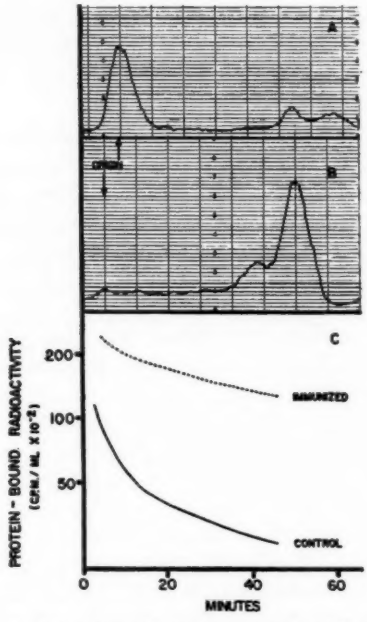


FIG. 5. As in Fig. 4. Animal passively immunized with anti-insulin serum from group 2. Other animals immunized with group 2 serum behaved in an identical manner.

30-minute period, through the catheter which was later used for blood sampling. At the end of the infusion, a sample was taken for measurement of antibody titer, and an insulin disappearance test was carried out. It is seen (Table II; Fig. 4) that the guinea pig immunized with group 1 serum was

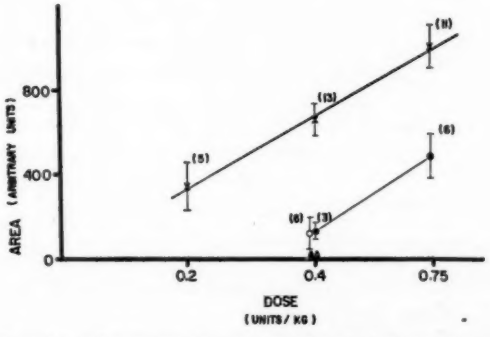


FIG. 6. Results of insulin responsiveness test in control and immunized animals. Values represent mean ± S.E. Number of animals in parentheses. × Control; ● passively immunized with serum from group 2; △ individual animals, actively immunized, group 1; ○ actively immunized, group 2.

able to bind all the insulin administered, yet the insulin disappeared from the circulation at a normal rate. When serum from the second group of actively immunized animals was infused, there was a distinct decrease in the rate of insulin disappearance (Table II, Fig. 5).

(*c*) *Effect of Active and Passive Immunization on Insulin Responsiveness*

Insulin responsiveness tests were performed on actively immunized guinea pigs from groups 1 and 2, and on guinea pigs passively immunized with serum from group 2. The results (Fig. 6) demonstrate a distinct decrease in the hypoglycemic response to insulin in all animals tested.

Discussion

Several workers have shown that guinea pig anti-insulin serum may interfere with the biological effect of insulin injected simultaneously into animals of other species (8, 9). The present results show, as expected, that the immunized guinea pig is also less responsive to insulin of the antigenic species. Since passively immunized animals also showed a decreased response, it is evident that the serum and presumably the circulating antibodies are, at least in large part, responsible for this effect.

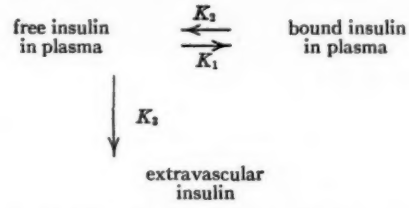
The above results also demonstrate that the presence of titers of antibodies sufficiently high to bind administered insulin completely need not result in delayed removal of the insulin from the circulation. When antiserum from an animal showing a normal insulin disappearance rate was used for passive immunization, the recipient animal continued to show the association of insulin binding by circulating antibodies and normal removal of insulin from the serum. Similarly, when serum from animals with delayed insulin disappearance was used for passive immunization, the recipients also showed this effect. This indicates that the delay in insulin disappearance is due to serum factor(s) rather than to tissue factor(s).

There are two possible explanations for these results: (i) Berson and Yalow have shown that antibodies differ in the rates of association with, and dissociation from, insulin, and indicated that the rate of dissociation may determine the availability of insulin to the tissues and consequently its hypoglycemic action (10, 11).

When insulin is incubated in vitro with antiserum, there occurs a reversible reaction in which insulin and antibody combine to form a complex:

$$\text{free insulin} \underset{K_1}{\overset{K_2}{\rightleftharpoons}} \text{bound insulin}$$

If at equilibrium the concentrations of insulin and antibody are such that virtually all the insulin is bound, then the fractional rate K_2 must be very small compared to K_1. When such relative concentrations are obtained by the injection of insulin in vivo, the same relationship must presumably obtain. Here, however, there is an added factor, disappearance of insulin from the plasma:



Now the fate of insulin is dependent essentially on the ratio K_1/K_3. The higher the ratio, the slower would be the disappearance of insulin from the plasma. The lower the ratio, the higher would be the rate of removal of insulin, until it approached the normal rate. The rate of dissociation of the antigen–antibody complex, in these circumstances, is then unimportant in determining the distribution and effect of insulin. (ii) There remains the possibility that the antigen–antibody complex is removed from the plasma at different rates in different animals, and that a high disappearance rate of I^{131}-insulin in immunized animals merely indicates a high disappearance rate of insulin–antibody complex. The fact that animals with high antibody titers and normal insulin disappearance rates had decreased responses to insulin tends to support this explanation.

The reason for the difference in disappearance rate between the two groups of animals is not clear. The only differences of which we are aware were in the immunization procedure: the use of different batches of adjuvant and the degree of homogenization (hand mixing for the first group as opposed to mixing in an electric homogenizer). Regardless of the reason, however, the observation remains that normal disappearance rates may obtain despite complete binding of administered insulin.

It was previously found that passive immunization of rabbits with heterologous antiserum resulted in protein-binding of insulin but did not interfere with removal of insulin from the serum (4). The possibility existed that the normal rate of insulin disappearance was in fact due to rapid removal from the circulation of the foreign antibody and the associated insulin. In view of the similar findings in the present work with homologous antiserum, it is unlikely that the former results were related to the source of antiserum.

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SOME EFFECTS OF DIETARY THIOURACIL AND DIETARY IODINE SUPPLEMENTATION ON THE RESPONSES OF THE IMMATURE PULLET TO ESTROGEN[1]

R. H. COMMON, A. J. MOO-YOUNG, AND K. A. MCCULLY

Abstract

Sexually immature pullets were given 1.5 mg estradiol benzoate by intramuscular injection *on alternate days* over a 14-day period. The resultant increase of oviduct weight was enhanced by 70% when 1% thiouracil was included in the diet over the period of the injections. Supplementation of the diet with 1.0 mg iodine per lb did not modify this effect of dietary thiouracil on the response of oviduct to estrogen. Consideration of this result in conjunction with previous results shows that a necessary condition for this positive effect of thiouracil on response of the oviduct to estrogen is administration of the estrogen on alternate days.

The following effects of thiouracil on the estrogenized pullets also attained statistical significance: decrease of estrogen-induced hypercalcemia; increase of ovary weight, liver weight, and total liver crude protein and fat; increase of kidney weight and percentage dry matter in kidney.

The iodine supplement increased ovary weight significantly in the unestrogenized pullets but not in the estrogenized pullets. The iodine supplement also tended to increase total liver crude protein in the estrogenized pullets but not in the unestrogenized pullets.

Introduction

Dietary thiouracil has been observed to enhance the hypertrophy of the oviduct of the pullet that follows upon estrogen treatment (1), but subsequent experiments have suggested that this effect of thiouracil is produced only under rather narrowly defined experimental conditions (2). These conditions appeared to include (*a*) use of a high level of dietary thiouracil; (*b*) introduction of the dietary thiouracil at the same time as the estrogen treatment is begun; and (*c*) administration of the estrogen by intramuscular injection every other day instead of every day. Commercial 'starter' rations of unknown iodine contents were used in both the experiments where dietary thiouracil increased the effect of estrogen on the oviduct, and hence it remained possible that level of dietary iodine might have been implicated. It became desirable, therefore, to repeat the original experiment (1) with a diet compounded so as not to be high in iodine and with the same diet supplemented with iodine. The experiment also provided a further test of the reproducibility of the effect observed in the original experiment (1) and an opportunity of extending the original observations to an examination of possible concurrent effects on liver nucleic acids and serum calcium levels.

[1]Manuscript received June 10, 1960.
Contribution from the Faculty of Agriculture of McGill University, Macdonald College, Que.
Macdonald College Series No. 480.

Experimental

General Technique and Analytical Methods

Forty-two crossbred pullets (Rhode Island Red X Barred Rock) were placed in individual cages. The birds were 9 weeks of age. The basal diet was made up so that its iodine content would not be unduly high. It contained corn meal 25, ground wheat 27, ground oats 20, wheat shorts 5, soybean oil meal 18, dried brewers' yeast 5, salt 0.5, commercial dicalciumphosphate 2. To each 100 lb was added 6 g manganese sulphate, 200,000 I.U. vitamin A, and 10,000 I.U. vitamin D. Both vitamins were incorporated as 'dry' preparations in order to avoid the use of fish oils.

The iodized diets were made up in the same way except that 1.0 mg iodine per lb was incorporated into the mixture in the form of sodium iodate. The nutritional requirement of the fowl for iodine is estimated at 0.2 mg iodine per lb feed for growing chickens or laying hens, and at 0.5 mg iodine per lb for young chicks or breeding stock (3). The iodine supplement was of itself sufficient, therefore, to provide somewhat more than the total requirement of iodine. Food consumption was limited slightly below appetite so that all birds would consume the same weight of food during the experiment.

All the birds were given the basal diet for a pre-experimental period of 14 days to accustom them to the experimental conditions. They were then allotted at random into six groups which were treated as follows:

Group	Diet	Hormonal treatment
A1	Basal	Nil
A2	Basal	1.5 mg ODB* every other day
A3	Basal + 1% thiouracil	1.5 mg ODB every other day
B1	Iodized	Nil
B2	Iodized	1.5 ODB every other day
B3	Iodized + 1% thiouracil	1.5 ODB every other day

*Estradiol benzoate, administered as Progynon B (Schering) by intramuscular injection.

The experimental treatments extended over a period of 14 days.

At the end of the experimental period, blood samples were drawn and the birds were then decapitated and allowed to bleed freely from the neck vessels. Ovaries, oviducts, thyroids, livers, and kidneys were removed and weighed. Serum calcium was determined by deproteinizing the serum with trichloracetic acid and titrating a suitable sample of the filtrate with ethylenediamine tetraacetate (4). Tissue powders were prepared from liver by a method described elsewhere (5) and analyzed for RNAP and DNAP by the methods of Ceriotti (6, 7). Kidney dry matter was determined by drying in the vacuum oven at 100° C. Liver crude protein (total N × 6.25) was determined by the macro-Kjeldahl method. Thyroids were fixed in Bouin's fluid, stored in 70% v/v ethanol, sectioned in paraffin wax, and stained with hematoxylin-eosin.

The average experimental results are presented in Table I. Two series of

TABLE I

Effects of (*a*) dietary iodine supplementation and (*b*) dietary thiouracil (1%) on responses of immature pullets to estrogen injected on alternate days
(Average results, seven pullets per group)

Group	A1	A2	A3	B1	B2	B3
Dietary iodine supplement, mg/lb	Nil	Nil	Nil	1.0	1.0	1.0
Dietary thiouracil, %	Nil	Nil	1.0	Nil	Nil	1.0
Total estradiol benzoate, mg	Nil	7×1.5	7×1.5	Nil	7×1.5	7×1.5
Live weight, initial, g	1207±27	1207±27	1196±14	1199±27	1224±17	1201±23
Live weight, final, g	1296±24	1300±25	1295±17	1281±23	1290±14	1289±12
Live weight, increase, g	89±4	92±8	99±8	76±12	66±8	89±16
Food consumed, kg	0.80	0.80	0.80	0.80	0.80	0.80
Ovary, mg	270±12	256±12	282±34	356±18	236±14	331±38
Oviduct, g	0.17±0.02	10.0±0.84	18.2±1.41	0.19±0.04	9.7±0.55	15.4±4.09
Thyroid, mg	113±13	110±17	95±7	124±12	98±11	98±7
Serum Ca, mg/100 ml, 4 days	11.4±0.21	15.8±0.81	12.8±0.63	11.4±0.15	15.3±0.69	12.8±0.27
Serum Ca, mg/100 ml, 14 days	11.4±0.16	35.6±6.5	25.4±3.8	11.3±0.17	38.7±5.0	19.1±1.8
Liver weight, g/kg live weight	13.7±0.52	18.9±0.76	24.7±1.08	14.0±0.56	20.3±1.09	26.1±1.14
Liver crude protein, g/kg live weight	2.96±0.13	3.92±0.13	4.93±0.23	3.03±0.14	4.07±0.16	5.51±0.20
Liver fat, g/kg live weight	0.51±0.02	0.77±0.05	0.91±0.03	0.54±0.02	0.80±0.08	1.00±0.05
Liver DNAP, mg/kg live weight	3.4±0.42	4.1±0.61	4.9±0.55	4.2±0.30	4.8±0.57	4.7±0.71
Liver RNAP, mg/kg live weight	10.3±0.52	15.0±0.06	16.6±0.92	10.9±0.80	17.3±1.21	19.9±1.40
Liver RNAP:DNAP	3.2±0.3	4.2±0.5	3.7±0.5	2.6±0.1	3.8±0.3	4.4±0.5
Kidney weight, g/kg live weight	4.1±0.17	4.8±0.30	6.7±0.27	4.6±0.14	4.6±0.11	6.8±0.38
Kidney dry matter, %	22.2±0.05	22.3±0.03	21.5±0.02	22.0±0.04	23.3±0.03	21.5±0.02
Kidney dry matter, g/kg live weight	0.91±0.04	1.08±0.07	1.43±0.05	1.01±0.03	1.07±0.03	1.45±0.09

NOTE: Figures preceded by ± sign are standard deviations of means.

possible effects were of interest in relation to the objects of the experiment. The first series comprised the possible effects of the dietary iodine supplementation on the response of the pullet to estrogen in the absence of thiouracil. The relevant comparisons involved groups A1, A2, B1, and B2. The second series comprised the possible effects of thiouracil and of iodine on the estrogenized pullets, or, in other words, on the response of the pullets to estrogen. The relevant comparisons involved groups A2, A3, B2, and B3 and constituted the comparisons that were of major interest to the present study. The two series of comparisons were considered separately as described below.

(A) Effect of Iodine on Response to Estrogen in Absence of Thiouracil Treatment

The data for groups A1, A2, B1, and B2, excepting the data for serum calcium, were subjected to an analysis of variance as follows:

Comparison	Degrees of freedom
Levels of iodine	1
Levels of estrogen	1
Interaction of iodine and estrogen	1
Error	24
Total	27

The results of these analyses of variance are set out in Table II in terms of the "*F*" values for the various comparisons. Significance is indicated in accordance with the usual convention, viz., non-significant $P > 0.05$, significant $P < 0.05$ and > 0.01, highly significant $P < 0.01$.

A tendency for the iodine supplement to reduce live weight increase did not

TABLE II

Analyses of variance for effects of estrogen and of iodine supplementation on immature pullets. Estrogen as 1.5 mg estradiol benzoate on alternative days for 14 days (Results stated as "*F*" values for each comparison)

	Iodine vs. no iodine	Estrogen vs. no estrogen	Interaction estrogen × iodine
Live weight, initial, g	0.04	0.27	0.26
Live weight, final, g	0.32	0.08	0.13
Live weight, increase, g	3.84	0.00	0.01
Ovary, mg	2.36	14.96**	8.45**
Thyroid, mg	0.00	1.67	1.13
Liver weight, g/kg live weight	1.12	55.12**	0.47
Liver crude protein, g/kg live weight	0.60	50.09**	0.07
Liver fat, g/kg live weight	0.31	29.56**	0.00
Liver DNAP, mg/kg live weight	2.19	1.85	0.04
Liver RNAP, mg/kg live weight	1.45	22.03**	0.51
Liver RNAP:DNAP	2.85	11.41**	0.05
Kidney weight, g/kg live weight	0.34	3.22	2.91
Kidney weight dry matter, %	1.31	3.93	2.21
Kidney weight dry matter, g/kg live weight	1.00	6.84**	1.28

**Highly significant.

attain significance at $P = 0.05$ during the experimental period of 14 days. Estrogen did not affect the live weight increase.

The iodine supplement increased ovary weight significantly in the unestrogenized bird, since a comparison of the ovary weights of groups A1 and B1 gave $t = 2.99$ for 12 degrees of freedom. The iodine supplement did not have this effect on the estrogenized birds (averages for groups A2 and B2 were not significantly different by 't' test). The high significance of the effects of estrogen on ovary weight, as shown in the analysis of variance (Table II), was due mainly to the positive effect of the iodine supplement on the ovary weight of the unestrogenized pullets; and the high significance of the interaction arose from the fact that the iodine supplement did not affect the ovary weight of the estrogenized birds. Iodine did not affect the thyroid weight nor did it produce any evident changes in the histology of the thyroids in these comparisons.

In agreement with many previous observations, estrogen increased liver weight, liver crude protein, liver fat, liver RNAP, and the ratio RNAP/DNAP in the liver. The average values suggested that estrogen had increased slightly the liver DNAP per kg live weight. Previous work has shown that estrogen tends slightly to increase liver DNAP per kg live weight (8, 9), but the effect observed in the present experiment was not statistically significant. Estrogen is also known to increase kidney weight (8) but this effect did not attain significance in the present experiment, although the estrogen treatment did increase significantly the percentage dry matter in the kidney.

Estrogen did not affect either the weight or histological picture of the thyroids. Experiments on the influence of estrogen on the thyroid, however, have given evidence of stimulation or depression according to the conditions of the experiment (10, 11).

The analysis of variance as outlined above was not applicable to the data for serum calcium because of their obvious non-homogeneity of variance. The necessary 't' tests between appropriate pairs of groups proved the high significance of the effect of estrogen and demonstrated that iodine had not affected the response of serum calcium to estrogen in the absence of thiouracil.

(B) Effects of Thiouracil and Iodine Supplementation on Estrogenized Pullets

The effects of thiouracil and of iodine supplement on the estrogenized pullets were examined by analyses of variance of the data for groups A2, A3, B2, and B3 as follows:

Comparison	Degrees of freedom
Levels of iodine	1
Levels of thiouracil	1
Interaction of iodine and thiouracil	1
Error	24
Total	27

The results of these analyses of variance are presented in Table III in terms of the "F" values for the various comparisons.

TABLE III

Analyses of variance for effects of dietary iodine supplementation and dietary thiouracil on the immature pullet injected with 1.5 mg estradiol benzoate on alternate days for 14 days (Results stated as "*F*" values for each comparison)

	Iodine vs. no iodine	Thiouracil vs. no thiouracil	Interaction iodine × thiouracil
Live weight, initial, g	0.26	0.65	0.07
Live weight, final, g	0.19	0.02	0.01
Live weight, increase, g	3.04	1.87	0.53
Ovary, mg	0.30	4.94*	1.63
Oviduct, g	1.95	41.15**	1.41
Thyroid, mg	0.29	0.82	0.80
Serum Ca, 4 days	0.16	23.62**	0.22
Serum Ca, 14 days	0.98	8.17**	0.81
Liver weight, g/kg live weight	1.54	28.49**	0.00
Liver crude protein, g/kg live weight	3.92	44.06**	1.39
Liver fat, g/kg live weight	0.81	6.49*	0.29
Liver DNAP, mg/kg live weight	0.14	0.26	0.40
Liver RNAP, mg/kg live weight	3.80	1.76	0.00
Liver RNAP:DNAP	7.84**	2.94	22.94**
Kidney weight, g/kg live weight	0.04	49.38**	0.32
Kidney dry matter, %	3.94	28.53**	3.94
Kidney dry matter, g/kg live weight	0.03	33.34**	0.06

* Significant.
** Highly significant.

(*i*) *Live Weights*

None of the group differences in initial or final live weight attained significance. A tendency for iodine supplementation to reduce live weight gain did not attain significance.

(*ii*) *Ovary Weight*

As already mentioned, iodine supplementation increased the ovary weight of the unestrogenized birds, but it had no such effect on the estrogenized birds (comparison of groups A1 and B1 and of A2 and B2). So far as comparison of groups A2, A3, B2, and B3 are concerned, iodine did not affect ovary weights but thiouracil increased ovary weight significantly. In point of fact, the ovary weights, and their trends, in groups B2 and B3 closely resembled those observed previously (1) for similar treatments, except that the differences did not attain significance in that previous work.

(*iii*) *Oviduct Weights*

The iodine supplement did not alter the responses of the oviduct to estrogen. The thiouracil treatment enhanced the response of the oviduct to estrogen to a highly significant degree and by almost 70%. This observation confirmed completely the original observation on this point (1). It would seem to be established, therefore, that the curious effect of thiouracil in enhancing estrogen-induced hypertrophy of the oviduct is contingent on the administration of the estrogens on alternate days and that it is independent of moderate variation in iodine content of the diet. In similar experiments where comparable total amounts of estrogen have been distributed over daily doses, no such effect of a high level of dietary thiouracil has been observed (2, 5).

(iv) Thyroid Weights

None of the group differences in thyroid weight attained significance. However, the effect even of high levels of thiouracil on thyroid weight are not usually evident within a period as short as 14 days. Histological examination of the thyroids gave clear evidence of the effect of thiouracil treatment. The follicles of groups A2 and B2 were well-defined and filled with colloid. There was no evidence that supplemental iodine had affected the histological picture presented by group B2 as compared with group A2. However, nearly all the follicles of group A3 had lost their colloid, except for one bird, and there was much hypertrophy of the follicular epithelium. This picture was consistent with inactivation of the thyroids of group A3. Group B3 presented a similar but possibly less extreme picture, so that the iodine supplement may have lessened slightly the antithyroidal effect of thiouracil.

(v) Serum Calcium

Estrogen-induced hypercalcemia was reduced highly significantly by the thiouracil treatment as early as the 4th day. This effect was still significant at 14 days. The iodine treatment did not modify the hypercalcemia significantly. This depressant effect of the thiouracil treatment fully confirmed previous results and conclusions regarding the conditions under which thiouracil will depress estrogen-induced hypercalcemia (2). The serum calcium levels of several of the birds in groups A3 and B3 were only slightly higher than average levels observed for the control groups A1 and B1. The early incidence of the effect on hypercalcemia is consistent with the view that thiouracil was stimulating the thyroid in the early stages of the experiment.

(vi) Liver Weight and Composition

Thiouracil increased liver weight per kg live weight to a highly significant degree. Iodine supplementation tended to *increase* this effect, although the effect did not quite attain significance at $P = 0.05$. Thiouracil greatly increased liver crude protein per kg live weight, in confirmation of previous observations (2, 13). Iodine supplementation *augmented* this effect to an extent that approached significance at $P = 0.05$. Liver fat was increased significantly by the thiouracil treatment but not by the iodine supplementation. The fat content of the liver was not excessive; this was probably related to the limitation of food intake, for thiouracil can lead to very high liver fat contents when food intake is high (2).

(vii) Liver Nucleic Acids

Neither iodine supplementation of the diet nor the thiouracil treatment had any significant effect on the liver DNAP of the estrogenized pullets. This observation was in agreement with previous results (2), where thiouracil also increased the liver weight of estrogenized pullets without significantly increasing the liver DNAP per kg live weight. Thiouracil of itself increases liver weight, liver crude protein, and liver RNAP, and these effects are additive with the similar effects of estrogen (2). Comparisons of the average values for

liver RNAP for groups A2 and B2 with the average values for groups A3 and B3, respectively, in the present experiment suggests that the thiouracil treatment had increased the liver RNAP of the estrogenized bird. This effect of thiouracil was far from attaining statistical significance (see Table III) in spite of the very highly significant effect of thiouracil on the liver crude protein of the estrogenized pullets. We are inclined, nevertheless, in view of previous results, to regard the effects of thiouracil on the average liver RNAP of the estrogenized pullets in the present experiment as real though of low statistical significance.

Increases of liver protein and liver RNAP consequent upon estrogen treatment can be related to hypertrophy of the liver cells and activation of the synthesis by the liver of lipophosphoprotein material that is passed into the plasma (8, 14). In the normal puberal pullet these materials will pass from the plasma to the ripening yolks in the ovary. The increase of liver crude protein and liver RNAP evoked by thiouracil treatment is of a different nature, insofar as there is no such concurrent stimulation of synthesis of plasma lipophosphoprotein (2). The precise reason why thiouracil increases liver crude protein and liver RNAP is not obvious.

The iodine supplement increased liver RNAP per kg live weight of the estrogenized pullets, and although the effect did not quite attain significance at $P = 0.05$, it suggests that the basal ration used in the present experiment was below optimal in respect of iodine content. The iodine treatment increased the ratio RNAP:DNAP of the liver to a highly significant degree, a point which also suggests that the effect on liver RNAP per kg live weight was real.

(*viii*) *Kidney Weight and Kidney Dry Matter*

Iodine supplementation had no effect on the kidney weight of the estrogenized pullets but the thiouracil treatment gave a great and highly significant increase of kidney weight. Thiouracil and estrogen are both known to increase kidney weight and their effects are additive (2). These effects of iodine and thiouracil were closely reflected in kidney dry matter per kg live weight, but iodine did increase kidney dry matter percentage.

General Discussion

The results now reported provide rather conclusive evidence that a high level of dietary thiouracil, begun at the same time as estrogen administered *every other day*, will enhance greatly the degree of hypertrophy of the oviduct induced by estrogen. These conditions also result in a depression of estrogen-induced hypercalcemia at both the 4th and 14th day of treatment. The results show in addition that the foregoing effects are largely independent of moderate variation in the iodine content of the diet.

It was suggested originally that this effect of thiouracil might be due to impairment of the functions of the liver in estrogen inactivation (1), but reasons have been adduced elsewhere against this hypothesis (2). The effect may possibly reflect a transient stimulation of thyroidal action by thiouracil.

Such a transient stimulation would be consistent with the effects on estrogen-induced hypercalcemia, because injected thyroxine (15, 16) and also dietary thyroid (17) depress estrogen-induced hypercalcemia. However, it is not easy to bring this hypothesis into line with the fact that the effect on the oviduct is not observed if similar total amounts of estrogen are administered in daily doses (2).

Histological examination suggested that the thiouracil had largely inactivated the thyroids by the end of the experiment, although the thyroids had not undergone any increase in weight in consequence of thiouracil treatment. Inactivation of the thyroids may have proceeded a little further in the absence of supplementary iodine than in its presence, and the enhancement of oviduct hypertrophy was apparently greater in group A3 (no iodine supplement) than in group B3 (iodine supplement), although this latter difference did not attain significance.

The significant effect of the iodine supplement on the ovary weight of otherwise untreated birds suggests that the iodine content of the basal ration was somewhat inadequate, a view that would fit in with the evidence that the iodine supplement stimulated increase of liver crude protein, liver RNAP, and the ratio RNAP: DNAP in estrogenized birds.

The results for the effects of thiouracil on the liver nucleic acids confirm previous observations. In addition, they show that the effects of thiouracil on the responses of liver nucleic acids and crude protein to estrogen are much the same when the estrogen is given on alternate days as when it is given daily. This contrasts with the observation that the effects of thiouracil on the response of the oviduct to estrogen are closely dependent on the distribution of the estrogen treatment, since the positive effect has only been obtained when the estrogen has been injected on alternate days. At the same time, the fact that such an effect is observable under certain conditions does suggest that thyroidal influences may play a role in connection with normal hypertrophy of the oviduct. Normal hypertrophy of the oviduct probably involves the concurrent action of estrogen, androgen, progestin, and thyroid hormone, and the balance between the influences of these agents.

Acknowledgments

The authors wish to express their thanks to the National Research Council of Canada for the grant-in-aid of research which made the work possible, and to the Schering Corporation, Montreal, for generous gifts of hormonal preparations. They wish also to thank their colleagues, H. F. MacRae, H. M. McConnell, and Chi-Ching Mok, for co-operation in various phases of the experimental work, and Professor H. A. Steppler for advice on statistical matters. They are further indebted to the Department of Pathology, McGill University, for preparation of sections and to Professor C. P. Leblond, Department of Anatomy, McGill University, for helpful advice.

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ZONE ELECTROPHORESIS OF PROTEIN-BOUND PHOSPHORUS OF FOWL'S SERUM[1]

K. A. McCULLY AND R. H. COMMON

Abstract

The movement of protein-bound phosphorus on zone electropherograms of fowl sera has been studied (*a*) by use of reactions for ester-linked protein phosphorus (ELPP) and (*b*) by use of sera in which the phosphorus had been labelled with P^{32}. Serum from estrogenized pullets yielded two major lipoprotein zones, P-1 and P-2, on electrophoresis in veronal buffer pH 8.6. Most of the ELPP or protein-bound P^{32} was associated with these two zones, although minor amounts of protein-bound P^{32} were detected in other zones except the albumin zone. Somewhat similar results were obtained with borate buffer pH 9.0.

Use of veronal–citrate buffer pH 8.6, or pretreatment of the serum with ethylenediaminetetraacetate, or removal of the serum calcium by oxalate precipitation abolished the resolution of two lipoprotein zones, which were replaced by a single lipoprotein zone. At the same time most of the ELPP or protein-bound P^{32} moved to a zone just ahead of the albumin zone.

It is suggested that chelation or removal of the serum calcium led to dissociation of phosvitin from the lipoproteins of the serum and the independent migration of this phosvitin to a zone slightly ahead of the albumin zone.

The pre-albumin zones of sera from chick embryos did not give any staining reaction for ELPP. This observation supports the conclusion that the pre-albumin zones of sera from embryos and of sera of estrogenized pullets or laying hens are due to different proteins, and that phosvitin is either absent from embryo serum, or present in amounts below the limit of sensitivity of the reaction used for its detection.

Introduction

Zone electrophoresis of egg yolk in veronal buffer has permitted the resolution of a zone that corresponded with phosvitin in respect of mobility and behavior on staining (1). A similar zone was distinguishable on zone electropherograms of crude 'lipophosphoprotein' obtained from the serum of laying or estrogenized hens by dilution precipitation (1). Comparable presumptive phosvitin zones could not be distinguished on similar electropherograms of whole serum from laying or estrogenized fowl, although phosvitin can be prepared from such sera (2, 3) and the mobility of phosvitin by itself is greater than that of serum albumin in veronal buffer pH 8.6. These observations suggested that the phosvitin fraction of serum might be associated with the lipoprotein fractions in ways not easily broken in the course of zone electrophoresis in veronal buffer. This prompted a study of the possibility of resolving a phosvitin zone on zone electropherograms of whole serum by use of veronal-citrate buffer, by pretreatment of the serum with ethylenediaminetetraacetate to chelate calcium, or by removal of calcium by oxalate. The effects of borate buffers were also studied, because Brandt *et al.* (4) and Clegg *et al.* (5, 6) have reported the presence of a component (Clegg's component 1)

[1]Manuscript received April 24, 1961.
Contribution from the Faculty of Agriculture of McGill University, Macdonald College, Que.
Macdonald College Journal Series No. 476.

in sera of laying hens or estrogenized fowl which moved ahead of the albumin zone (Clegg's component 2) in free electrophoresis in borate buffer pH 8.6.

The present paper describes the resolution of a presumptive phosvitin zone on electropherograms of whole sera from estrogenized pullets. The movement of this presumptive phosvitin zone has been followed by the use of stains for ester-linked protein phosphorus (ELPP) and also in greater detail by use of sera in which phosphorus had been labelled by P^{32}.

Experimental

Electrophoretic Apparatus

Zone electrophoresis was done in a horizontal Matthew cell (Matthew Laboratories, New York) as described previously (1). Whatman 3MM paper was cut into strips 7.0 in. wide and one such strip was used for each electropherogram. Serum samples were applied in a narrow transverse band across the strip near the cathodic end and at the rate of 400 μl per 7.0 inch width of paper. The current density was 0.57 ma per inch width of paper. The period of electrophoresis was usually 24 hours. The apparatus was kept in a room maintained at approximately 5° C.

Buffers

(*a*) Veronal buffer pH 8.6, $\mu = .0.05$. Prepared by dissolving 1.84 g diethylbarbituric acid and 10.30 g sodium diethylbarbiturate in water and making up to 1.0 liter volume.

(*b*) Borate buffer pH 8.6 (Brandt *et al.* (4)).

(*c*) Borate buffer pH 9.0 (Block *et al.* (7)).

(*d*) Veronal–citrate buffer pH 8.6 (Deutsch and Goodloe (8)).

Staining Methods

On completion of an electrophoretic run, each main strip was air-dried and then cut longitudinally into substrips, which were then stained for protein, or for lipid, or for ELPP as follows:

1. Protein was stained with Amidoschwarz-10B on substrips that had been freed from lipid by solvent extraction (1).

2. Lipid was stained with oil red O on untreated strips as described by Durrum *et al.* (9).

3. ELPP was stained on strips that had been freed from lipid. The method was either that of Hanes and Isherwood (10), or that of Wade and Morgan (11), or that of Harrap (12). The last method was found to be the most generally satisfactory, but the blue zones indicative of ELPP became paler after the final treatment with α-benzoinoxime, and the stained strips were not usually suitable for photography.

Counting Technique

Electrophoretic technique and buffers for the experiments with P^{32}-labelled sera were as described above. A substrip 0.75 in. wide was cut from the main electropherogram. The distribution of P^{32} on this strip was then measured by

means of a Forro radiochromatogram scanner (Forro Scientific Co., Evanston, Illinois) coupled to an automatic recorder (Esterline-Angus Company, Inc., Indianapolis, Illinois). The strip was then freed from lipid and scanned again to give the distribution of protein-bound P^{32}. The tracings were transferred to rectangular co-ordinates for presentation as figures.

Analytical Methods

Serum calcium was determined on trichloroacetic acid filtrates of the serum by titration with ethylenediaminetetraacetate (13). Sucrose was added before titration, as suggested by Bond and Tucker (14).

Serum inorganic phosphorus, acid-soluble phosphorus, and lipid phosphorus were determined by the standard procedures described by Hawk *et al.* (15), except that the determinations of phosphorus were done by the method of Martin and Doty (16). Serum total protein phosphorus was determined as described elsewhere (17).

Nomenclature

The various zones on the electropherograms were designated as in a previous communication (1). No particular merit is claimed for this provisional system. Only one lipoprotein zone was clearly resolved under some of the conditions used. The designation 'L' was used for the single zone observed under such conditions. Presumptive phosvitin zones were designated 'PT'.

Experiment 1. Reactivity of Phosvitin with Reagents for Ester-linked Phosphate

Electropherograms of phosvitins prepared from hen's serum and from egg yolk were run in veronal buffer pH 8.6. The phosvitin zones stained with Amidoschwarz-10B in the way that is characteristic of phosvitin, i.e., the dye tended to wash out from the stained zones rather readily whenever background staining was being removed. The phosvitin zones gave positive reactions with each of the three reagents for ELPP mentioned above.

Experiment 2. Zone Electrophoresis of Sera from Estrogenized Pullets in Different Buffer Systems

Two pullets aged 12 weeks were each given 1 mg estradiol benzoate ('Progynon B', Schering) per day for 5 successive days. Blood samples were withdrawn and the serum allowed to separate. The serum calcium level was 34.2 mg per 100 ml for one pullet and 31.9 mg per 100 ml for the other.

Electropherograms of these sera were run in the following buffers: (A) veronal pH 8.6, (B) borate pH 8.6, (C) borate pH 9.0, (D) veronal-citrate pH 8.6.

Representative results are presented in Fig. 1.

The protein resolution in veronal buffer (Fig. 1A) conformed in detail with resolutions previously reported (1). Two lipoprotein zones (P-1 and P-2) were resolved and showed the usual dense staining for lipid. Faint staining for lipid was present also on regions corresponding to the α_2-globulin and α_3-globulin, P-1 and P-2 zones gave strong reactions for ELPP but no reaction for ELPP was perceptible elsewhere.

Resolution of the protein zones in borate buffer pH 8.6 (Fig. 1B) was relatively poor. Only an albumin zone, a general globulin zone, and a single lipoprotein zone were clearly distinguishable. The lipoprotein zone stained heavily and faint diffuse staining for lipid extended from this zone as far as the trailing edge of the albumin zone. Some reaction for ELPP occurred in a zone (PT) just ahead of the maximum of the albumin region, but no reaction for ELPP was observed in the lipoprotein zone. The position of this ELPP zone suggested that it might be due to a component similar to Clegg's component 1 (4, 5, 6). The separation of the zone from albumin was relatively slight, so that the two zones overlapped. The use of borate buffer pH 8.6 was not studied further because of the relatively poor resolution of protein zones.

Borate buffer pH 9.0 gave resolution of protein zones (Fig. 1C) similar to that obtained with veronal buffer, with the exception that the lipoprotein zone was not resolved into P-1 and P-2 zones. In addition to heavy lipid staining of the single broad lipoprotein zone, there were faint zones of lipid staining corresponding to the α_2- and α_3-globulin zones. There was a reaction for ELPP in the lipoprotein zone, but an ELPP reaction could not be detected with certainty elsewhere. The latter observation was unexpected in view of the results with borate buffer pH 8.6, but it was confirmed in later work (see below).

Veronal–citrate buffer (Fig. 1D) gave clear resolution of an albumin zone and of two globulin zones. Only a single broad lipoprotein zone was observed. Staining for lipid gave the usual dense staining in the lipoprotein (L) zone and faint staining in zones corresponding with the α_2-globulin and the leading third of the albumin zone. No reaction for ELPP was perceptible in the lipoprotein zone, but there was a well-defined reaction for ELPP in a zone (PT) just ahead of the albumin peak. It is suggested that it was due to phosvitin liberated from its association with other proteins of the 'lipophosphoprotein' complex as a consequence of chelation of calcium by the citrate of the buffer. This presumptive phosvitin carried with it a small amount of lipid. It may be noted again that its position corresponded approximately with that taken by Clegg's component 1 in free electrophoresis of sera of hens or estrogenized fowl in borate buffer (4, 5, 6).

Experiment 3. Effects of (a) Pretreatment of Serum with Ethylenediaminetetraacetate (as Complexone III) and of (b) Removal of Calcium by Precipitation with Oxalate

If the appearance of a presumptive phosvitin (PT) zone in Figs. 1B and 1D were due to chelation of calcium, then somewhat similar results should follow pretreatment of the serum with EDTA or removal of calcium by precipitation with oxalate. Samples of serum from the estrogenized pullets and from an untreated cock were treated with complexone* (5 mg complexone III per ml serum) and then subjected to zone electrophoresis as follows:

*B. Siegfried Limited, Zofingen, Switzerland.

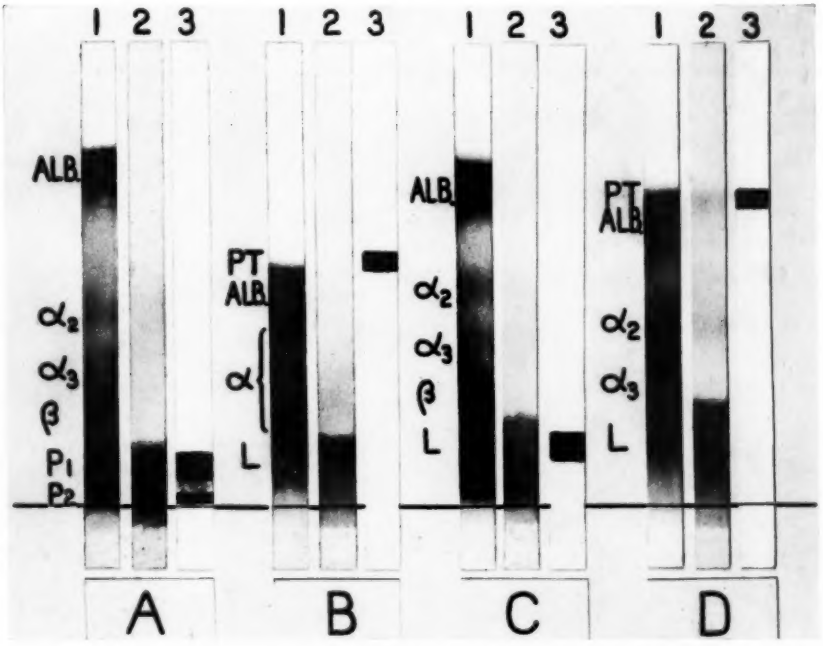

FIG. 1. Electropherograms of sera of estrogenized pullets in different buffers. Matthew cell. Temp. 5° C. Separate strips stained as follows: (1) Amidoschwarz-10B, (2) oil red O, (3) reagent of Harrap (12) for ester-linked protein phosphorus (ELPP). (A) Veronal buffer pH 8.6. (B) Borate buffer pH 8.6 (4). (C) Borate buffer pH 9.0 (7). (D) Veronal–citrate buffer (8).

Key to zones: PT = phosvitin; other protein zones designated as suggested by McCully *et al.* (1).

NOTE: Colors of strips stained for ELPP were unsuitable for photography, and position of reaction has been indicated by heavy pencil shading.

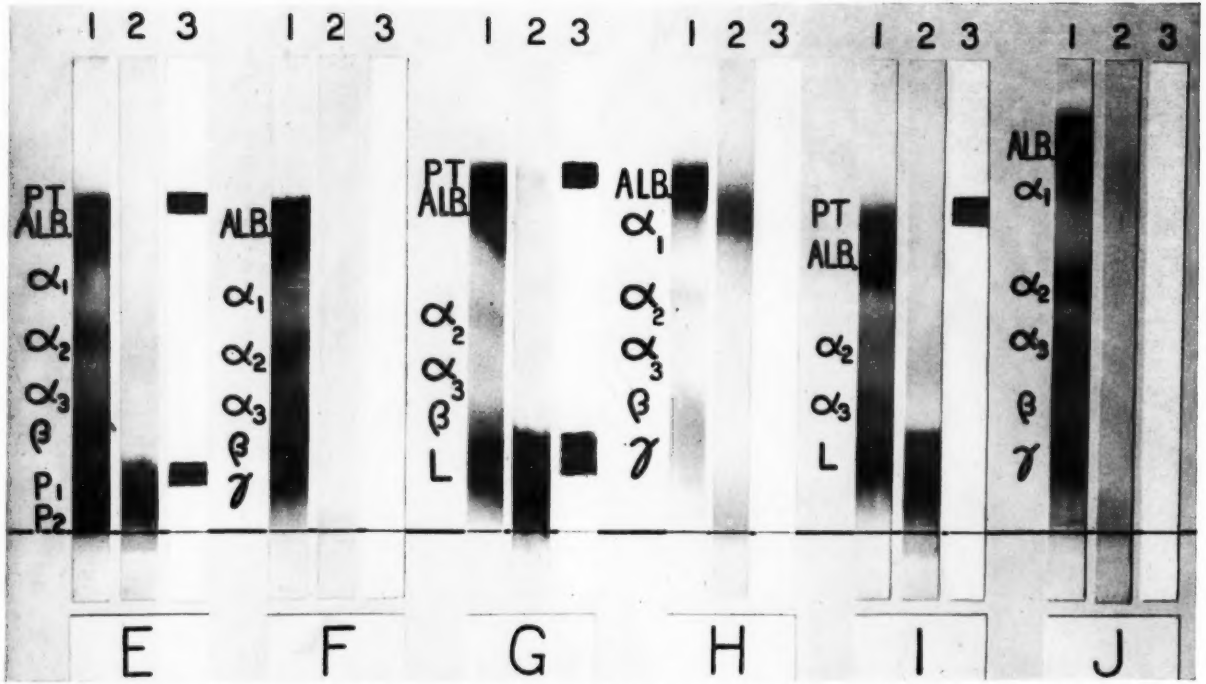

FIG. 2. Electropherograms of sera of estrogenized pullets and of a cockerel in different buffers with pretreatment of the sera with complexone III. Conditions and stains as for Fig. 1. (E) Pullet serum, veronal pH 8.6. (F) Cockerel serum, veronal pH 8.6. (G) Pullet serum, borate pH 9.0. (H) Cockerel serum, borate pH 9.0. (I) Pullet serum, veronal–citrate pH 8.6. (J) Cockerel serum, veronal–citrate pH 8.6.

McCully and Common—Can. J. Biochem. Physiol.

Strip	Serum	Buffer
E	Estrogenized pullet	Veronal pH 8.6
F	Cock	Veronal pH 8.6
G	Estrogenized pullet	Borate pH 9.0
H	Cock	Borate pH 9.0
I	Estrogenized pullet	Veronal–citrate pH 8.6
J	Cock	Veronal–citrate pH 8.6

The cock's serum was included in order to check possible effects of the complexone and different buffers on the resolutions ordinarily obtained with sera of cocks or unestrogenized immature pullets. The results for these complexone-treated sera are exemplified in Fig. 2.

Figure 2E shows that the results for staining of protein zones and lipid zones resembled those obtained in the absence of complexone, except that in some of the electrophoretic runs the resolution of P-1 and P-2 zones was abolished. Staining for ELPP showed the presence of reactive material (PT) just ahead of the albumin zone in strip E. This zone was not observed in strip A (Fig. 1) or in any runs in plain veronal buffer. The staining for ELPP in the lipoprotein region of strip E (Fig. 2) was fainter and was not resolved between two zones as compared with strip A (Fig. 1). These observations suggested that chelation of divalent ions by complexone had an effect similar to that of inclusion of citrate in the buffer in so far as both measures resulted in the appearance of an ELPP zone just ahead of the albumin zone, reduction or disappearance of ELPP staining in the lipoprotein zone, and a diminution or abolition of the resolution of two distinct lipoprotein zones, viz., of P-1 and P-2.

Figure 2F shows the pattern obtained when cock's serum was treated as had been the estrogenized pullets' serum for Fig. 2E. The resolution of protein zones was the same as that obtained without addition of complexone, and no reaction for ELPP was perceptible. This showed that the presence of complexone did not modify the electrophoretic behavior of the zones ordinarily resolved on electropherograms of sera from cocks or from immature pullets; hence the observed modifications of electrophoretic patterns by complexone were a function of the presence of the estrogen-induced proteins.

Figure 2G shows that pretreatment of the estrogenized pullet's serum with complexone followed by electrophoresis in borate buffer pH 9.0 gave essentially the same pattern as that shown in Fig. 2E, except that the resolution of P-1 and P-2 zones was completely abolished.

Figure 2H shows the pattern of the cock's serum treated as was the estrogenized pullet's serum exemplified in Fig. 2G. The results were substantially the same as for Fig. 2F and a similar description applies.

Figure 2I shows the results for complexone-treated serum run in veronal–citrate buffer. The results for protein resolution, lipid staining, and reaction for ELPP were substantially the same as those obtained by use of veronal–citrate alone, as shown in Fig. 1D. There was, however, fairly strong staining with Amidoschwarz just ahead of the albumin region and corresponding in

position with the zone of reaction for ELPP. This region also stained faintly but distinctly for lipid. Figure 2J shows the results for cock's serum treated as for Fig. 2I. The patterns were similar to those for Fig. 2, F and H.

Sera from which calcium had been removed by precipitation with oxalate gave results so closely similar to those obtained for sera pretreated with complexone that presentation of relevant figures was deemed unnecessary.

Experiment 4. Movement of P^{32}-Labelled Protein Phosphorus in Electrophoresis of Avian Sera

The observations described above showed that the movement of ELPP on electropherograms of avian sera may be greatly affected by the nature of the buffer, and especially by the presence or absence of chelating agents. There remained the possibility that minor amounts of protein-bound phosphorus might not have been detected by reason of insufficient sensitivity of the reaction for ELPP. Clegg *et al.* (18, 19) have shown that appreciable amounts of P^{32} are present through the region of protein migration in free electrophoresis of avian sera labelled with P^{32}, but their observations did not distinguish protein-bound P^{32} from lipid-bound P^{32}. These considerations prompted further experiments based on the use of P^{32}-labelled sera, because it is relatively easy to distinguish protein-bound P^{32} from total P^{32} on zone electropherograms.

Two pullets were each given 1.0 mg estradiol benzoate (Progynon B, Schering) per day for 5 days. In addition, P^{32}-labelled inorganic phosphate (0.5 millicurie) was injected intramuscularly each morning. On the 5th day, and 6 hours after the last injection, the pullets were bled and serum samples were separated. One other pullet was similarly injected with P^{32}-labelled phosphate but was not estrogenized. This bird served as a control. Some relevant analytical data for these sera are set out in Table I.

TABLE I

Analyses of sera from pullets treated with P^{32}-labelled orthophosphate so as to label serum lipid phosphorus and serum protein phosphorus

	Control pullet (unestrogenized)	Pullet No. 1 (estrogenized)	Pullet No. 2 (estrogenized)
Total crude protein (N × 6.25) g/100 ml	3.40	5.04	4.76
Serum Ca, mg/100 ml	12.4	36.7	24.4
Serum P, mg/100 ml			
Inorganic	6.1	14.9	12.5
Acid-soluble (total)	6.8	16.1	13.6
Lipid	9.8	56.1	29.7
Protein	0.31	14.0	6.8

The following six electrophoretic runs were made on each serum. (1) Veronal buffer pH 8.6. (2) Serum pretreated with complexone; veronal buffer pH 8.6. (3) Borate buffer pH 9.0. (4) Serum pretreated with complexone; borate buffer

pH 9.0. (5) Veronal–citrate buffer pH 8.6. (6) Serum pretreated with complexone; veronal–citrate buffer pH 8.6.

The experimental results for pullet No. 1 are presented in Figs. 3, 4, and 5.

The results for veronal buffer (Fig. 3) agreed with those obtained by staining for ELPP (Fig. 1A) in so far as there were two main protein-P^{32} zones near the

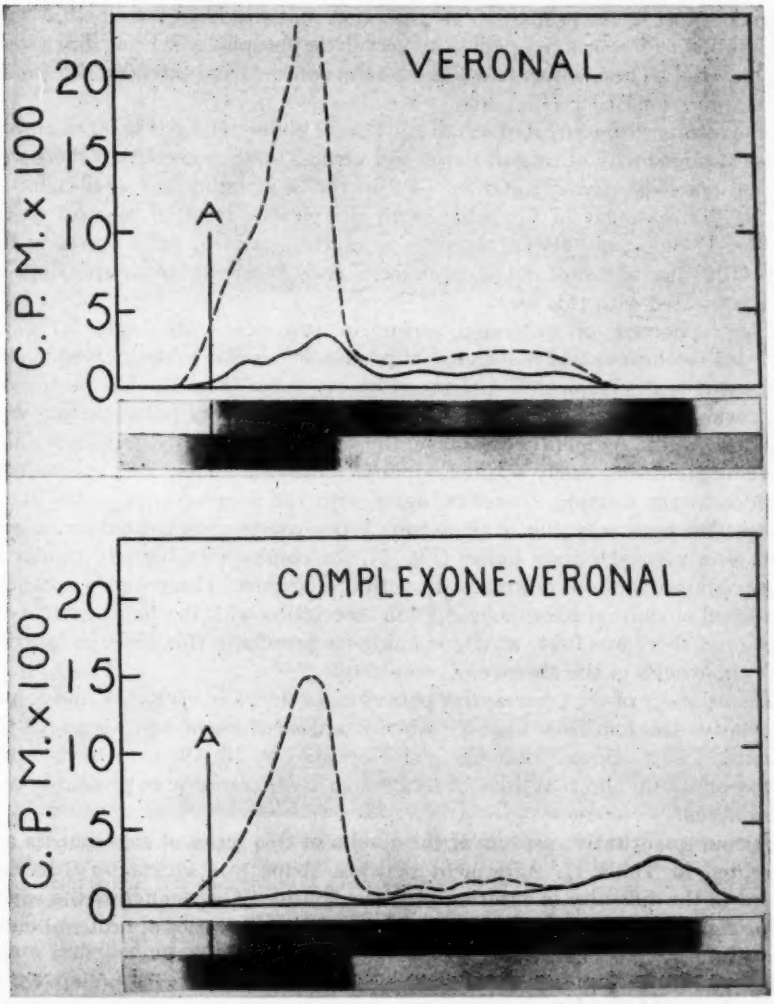

FIG. 3. Distribution of total (broken line) and protein-bound (solid line) P^{32} in electropherograms of serum of estrogenized pullet in veronal buffer pH 8.6 in absence and presence of complexone III. Conditions of electrophoresis as in legend of Fig. 1. Upper strips, Amidoschwarz-10B. Lower strips, oil red O.

point of application, but they further revealed that minor amounts of protein-P^{32} were present on the electropherogram as far as a point just short of the albumin zone. All this protein-P^{32} was accompanied by phospholipid-P^{32}, but there was no clear association of minor P^{32} peaks with individual globulin zones. Pretreatment of the serum with complexone did not appreciably modify the resolution of the protein zones as shown by staining, but it did result in a striking movement of most of the protein-P^{32} to a position that straddled the albumin zone. This protein-P^{32} was accompanied by very little phospholipid-P^{32} or lipid-staining material. These observations as a whole confirmed and extended those made by staining for ELPP (Fig. 2E).

The results with untreated serum and borate buffer pH 9.0 (Fig. 4) resembled those obtained with untreated serum and veronal buffer, except that there were indications of protein-P^{32} and lipid-P^{32} in the α_2-globulin and α_3-globulin regions. Pretreatment of the serum with complexone resulted in most of the protein-P^{32} moving forward to give a sharply defined peak (presumptive phosvitin) just ahead of the albumin zone. A slight amount of phospholipid-P^{32} was associated with this peak.

Electrophoresis of untreated serum in veronal–citrate buffer (Fig. 5) afforded resolutions and movement of protein-P^{32} similar to those given by pretreatment with complexone and use of borate buffer (Fig. 4). The protein-P^{32} peak was ahead of the albumin peak and gave only very faint staining with Amidoschwarz. As mentioned above, the dyestuffs ordinarily used in staining proteins are rather easily washed out from phosvitin zones. The results with Amidoschwarz staining, therefore, agree with the suggestion that the major protein-P^{32} peak was due to phosvitin. When complexone-treated serum was used with veronal–citrate buffer (Fig. 5), the results were broadly similar to those obtained without pretreatment with complexone. These were indications of a small amount of phospholipid-P^{32} in association with the main protein-P^{32} peak, and there was fairly strong staining for protein in this zone, in contrast with the results in the absence of complexone.

The behavior of the presumptive phosvitin, as shown in Figs. 3, 4, and 5, and its relative freedom from lipid-P^{32} when it appeared in, or just ahead, of the albumin region, suggest that Clegg's component 1 (18, 19) may have represented phosvitin and that most of its P^{32} may have been due to protein-bound phosphorus.

Certain quantitative aspects of the results of this series of experiments are presented in Table II. Agreement between strips was satisfactory, having regard to the difficulty of ensuring even distribution of serum across the entire strip. Agreement was very good, however, so far as the ratios of protein-bound P^{32} to total P^{32} were concerned.

The results with pullet No. 2 confirmed those obtained with pullet No. 1, except in so far as the levels of lipid-P and protein-P were lower (see Tables I and II). Diagrams of the scanning patterns have accordingly been omitted.

The results for the serum of the control pullet are presented in Fig. 6. The

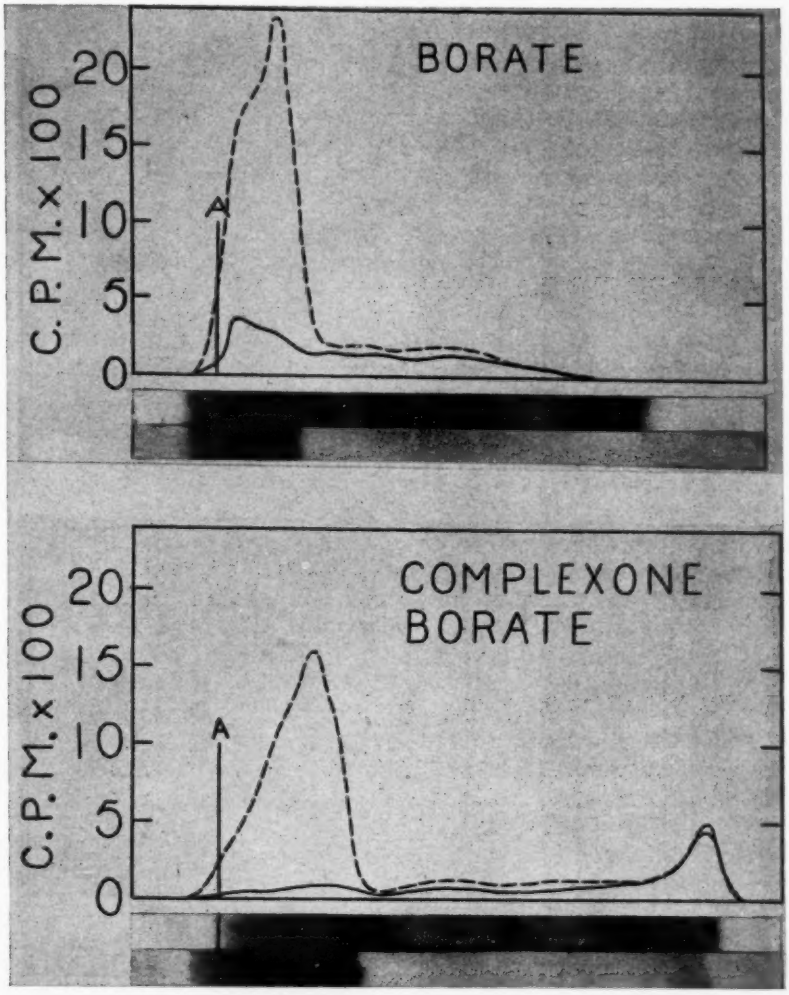

FIG. 4. Distribution of total (broken line) and protein-bound (solid line) P^{32} in electropherograms of serum of estrogenized pullet in borate buffer pH 9.0 in absence and presence of complexone III. Conditions of electrophoresis as in legend of Fig. 1. Upper strips, Amidoschwarz-10B. Lower strips, oil red O.

data provided no indication of phosphoprotein-P^{32} in four of the tests and only very small and doubtful traces in the remaining two tests. It was concluded that the protein-P content of the control serum was negligibly small, if any (see also Table I). Consequently, the radioactivity corresponded almost entirely with phospholipid-P^{32}. In each of the six electropherograms, there was a

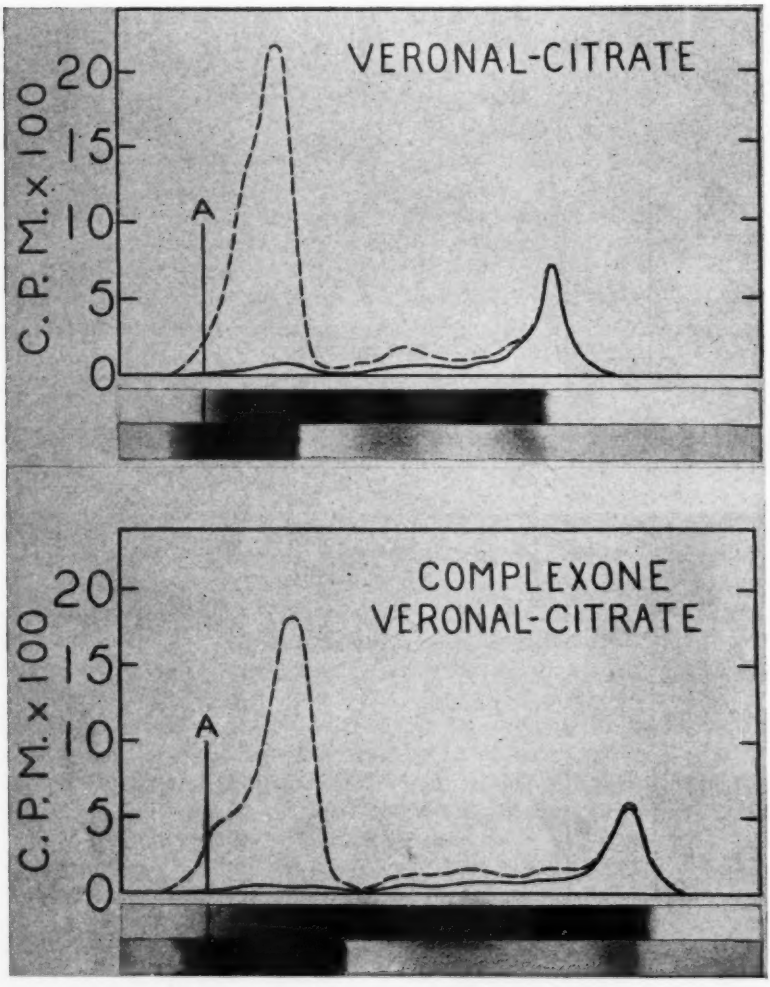

FIG. 5. Distribution of total (broken line) and protein-bound (solid line) P^{32} in electropherograms of serum of estrogenized pullet in veronal–citrate buffer pH 8.6 in absence and presence of complexone III. Conditions of electrophoresis as in legend of Fig. 1. Upper strips, Amidoschwarz-10B. Lower strips, oil red O.

minor phospholipid-P^{32} peak near the point of application and a major phospholipid-P^{32} peak just short of the albumin zone. The position of the major peak corresponded rather closely with the position of a maximum of staining with oil red O. The scanning patterns did not display marked differences as between the different buffer systems, but the sharpest resolution of the major phospholipid peak was obtained with veronal–citrate buffer, i.e., with the buffer which

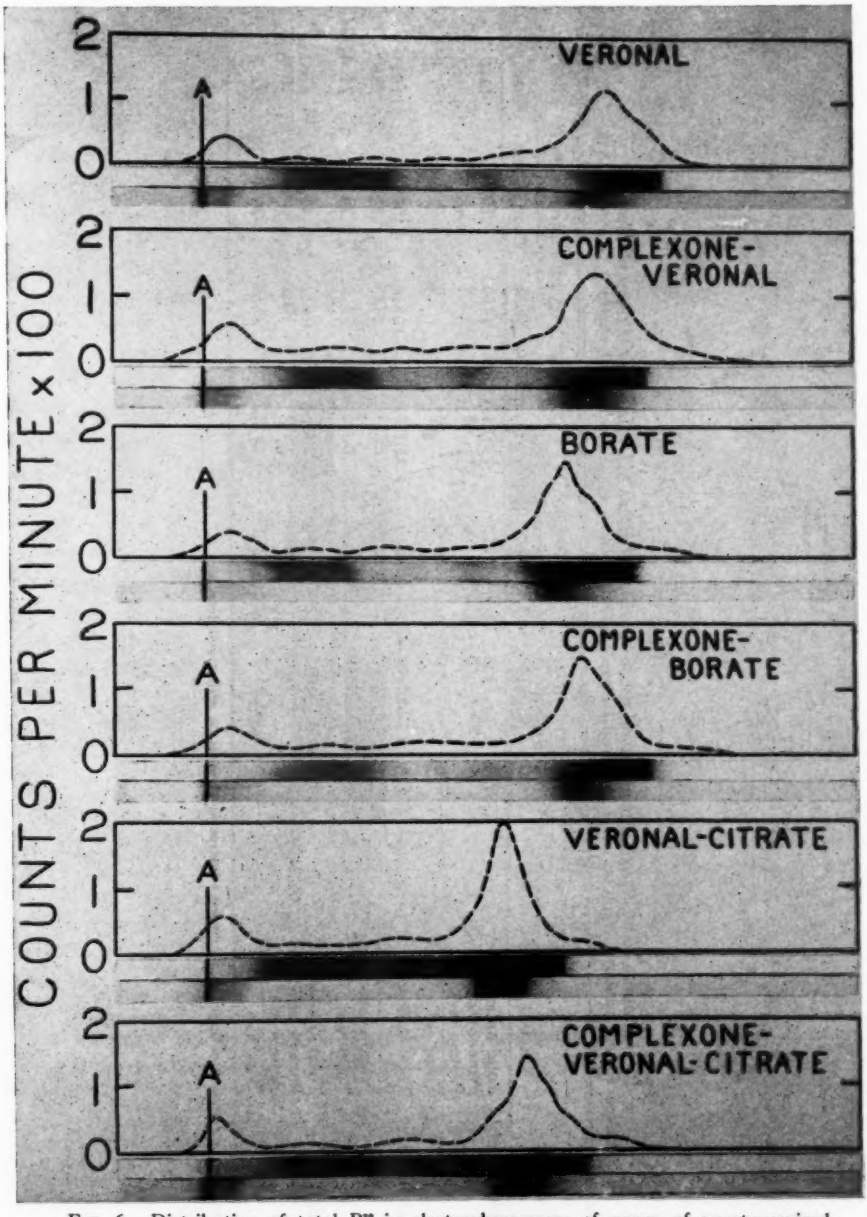

FIG. 6. Distribution of total P^{32} in electropherograms of serum of unestrogenized pullet in various buffers as indicated. Conditions of electrophoresis as in legend of Fig. 1. Upper strips, Amidoschwarz-10B. Lower strips, oil red O. (Slight but doubtful indications of protein-bound P^{32} noted on two only of the electropherograms were not inserted on the figures.)

TABLE II

Proportions of total P^{32} and protein-bound P^{32} on electropherograms of fowl's sera in different buffers.
Radioactivity expressed as c.p.m. per 0.75 in width strip equivalent to ca. 43 μl serum. Slit width on counter 0.25 in.

		Veronal buffer		Borate pH 9 buffer		Veronal–citrate buffer		
		None*	Complexone*	None	Complexone	None	Complexone	Average
Control pullet	Total radioactivity	1004	1408	1112	1153	1250	1033	1160
	Protein radioactivity	0	0	0	0	0	0	0
	$\frac{\text{Protein rad.}}{\text{Total rad.}} \times 100$	0	0	0	0	0	0	0
Pullet No. 1	Total radioactivity	15120	10494	11349	11075	10546	10795	11563
	Protein radioactivity	3168	2728	3230	2923	2435	2618	2850
	$\frac{\text{Protein rad.}}{\text{Total rad.}} \times 100$	21.0	26.0	28.5	26.4	23.1	24.3	24.6
Pullet No. 2	Total radioactivity	4931	4653	4966	4688	4822	4515	4763
	Protein radioactivity	1202	1102	1078	1117	1029	947	1079
	$\frac{\text{Protein rad.}}{\text{Total rad.}} \times 100$	24.4	23.7	21.7	23.8	21.3	21.0	22.7

*None = no pretreatment of serum. Complexone = pretreatment of serum with complexone.

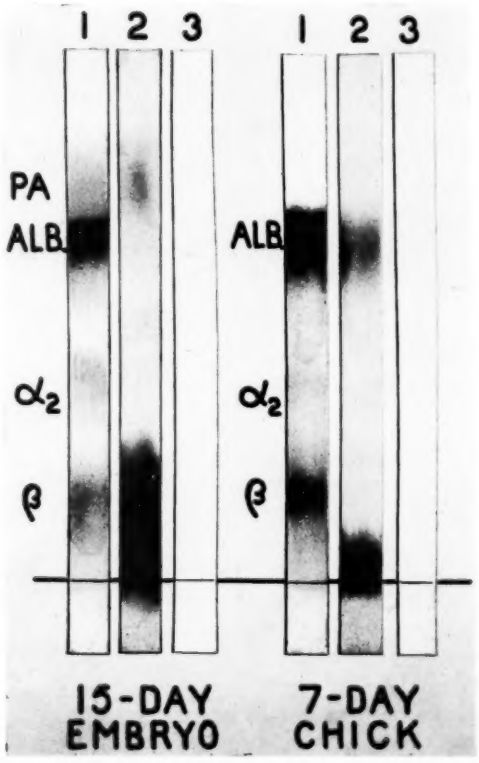

FIG. 7. Electropherograms of sera from 15-day embryo and from 7-day chick. Electrophoresis in veronal buffer pH 8.6. Staining as in Fig. 1. (Similar patterns were obtained whether or not the serum was pretreated with complexone III.)

gave the clearest resolution of protein-P in the cases of the sera of the estrogenized pullet.

Experiment 5. The Absence of ELPP from the Pre-albumin Components of Embryonic Fowl Serum

The serum of the chick embryo contains one or more components that move ahead of the albumin in free electrophoresis (20) or zone electrophoresis (21), whereas no such pre-albumin component is seen when sera of immature pullets or of cockerels are subjected to electrophoresis under the same conditions. Heim and Schechtman (20) concluded from their free electrophoresis studies that the pre-albumin components observed by them in embryonic sera were probably not identical with the pre-albumin components which they observed in sera of laying hens. Marshall and Deutsch (22) have reported that embryonic sera contain little or no protein-bound phosphorus. Absence of ELPP from the pre-albumin component of embryonic serum resolved in zone electrophoresis would provide some further evidence for the non-identity of pre-albumin components of embryonic sera with those of sera from laying hens.

Bulked samples of serum were obtained by heart puncture from chick embryos at 15 days and at 18 days of incubation, from day-old chicks and from 7-day chicks. The total serum protein ($N \times 6.25$) of these sera were determined by precipitation of the protein with trichloroacetic acid and micro-Kjeldahl determination of nitrogen in the precipitates. The values obtained were as follows:

Origin of serum sample	Total serum protein g per 100 ml
15-day embryos	1.17
18-day embryos	1.73
Day-old chicks	2.24
7-day-old chicks	2.16

The foregoing data agree closely with the values reported by Vanstone *et al.* (21) for comparable samples.

Each of these four samples was divided into two subsamples. One subsample was subjected to electrophoresis in veronal buffer pH 8.6. The other subsample was treated with complexone (3 mg complexone III per ml serum) and then subjected to electrophoresis in veronal buffer. It was found that pretreatment with complexone did not alter the electrophoretic pattern of these sera. Typical results for 15-day embryos and for 7-day old chicks are shown in Fig. 7. The resolutions corresponded closely with those reported by Vanstone *et al.* (21) for comparable sera so far as staining for protein and lipid were concerned. Staining for ELPP was uniformly negative. This negative result could be a consequence of lower protein concentrations, but against this it should be emphasized that we have obtained a positive ELPP reaction in every instance where we have applied the ELPP reagents to a phosvitin or presumptive phosvitin zone that stained as deeply with Amidoschwarz-10B as did the pre-albumin zones of

the embryo sera. It may be concluded that the pre-albumin zone of embryonic sera is not phosvitin and that it is different from the pre-albumin zones observed when sera from estrogenized fowl are resolved by use of suitable chelating agents or buffers. This conclusion is in agreement with the suggestions of Heim and Schechtman (20), and with the observations of Marshall and Deutsch (22).

Discussion

Ultracentrifugation is the best method for resolution of serum proteins in so far as it is the technique least liable to alter the proteins from their native states. Free electrophoresis involves the use of buffer solutions. Zone electrophoresis introduces additional complications associated with the actions of the supporting medium (23), although it is the most readily available technique of the three. It is highly desirable that some correlation between results from the three techniques should be available, but this problem is complicated by the appearance of the phospholipoprotein complex in the serum of the bird in response to estrogenic activity.

Schjeide and Urist (24) have distinguished two major ultracentrifugal protein components of egg yolk and have named these yolk X-1 phosphoprotein and yolk X-2 lipoglycoprotein. They have distinguished two analogous protein components in the serum of estrogenized fowl and have named these serum X-1 phosphoprotein and serum X-2 lipoglycoprotein. Cook and his collaborators have distinguished seven protein components in egg yolk, viz., lipovitellenin (25, 26), phosvitin, α-livetin, β-livetin, α-lipovitellin, β-lipovitellin, and γ-livetin (27). They have pointed out that β-lipovitellin and γ-livetin move as a single component in free electrophoresis in veronal buffer pH 9.0, $\mu = 0.3$. It is not yet certain how these various proteins are related to zone electrophoretic fractions, such as P-1 and P-2, of serum or yolk.

Yolk phosvitin may be separated from yolk lipovitellins by manipulation of the concentration of magnesium ions (28) and a serum phosvitin may be obtained by similar manipulation of the serum proteins of the estrogenized or laying fowl (2, 3). The present work has shown that serum phosvitin can probably be dissociated from the lipoprotein components of the serum of estrogenized fowl by zone electrophoresis when the conditions are such as to chelate calcium. When this resolution was achieved, the resolution of P-1 and P-2 lipoprotein zones was abolished as phosvitin was removed from the lipoprotein complex by electrophoresis. The precise reasons for the resolution of these two zones in zone electrophoresis of either yolk or serum in veronal buffer pH 8.6 calls for further study. The two zones appear not to be resolved in paper electrophoresis when the ionic strength of the buffer is increased to $\mu = 0.3$ by suitable addition of NaCl (unpublished observation).

Ericson *et al.* (29) and Clegg *et al.* (30) have demonstrated that the presence of calcium ions did not alter the number or order of the free electrophoretic components of normal cockerel serum, but that it reduced drastically the mobility of the leading component (Clegg's component 1) of sera from estro-

genized pullets. The results now reported are the converse of these observations, in so far as chelation of calcium led to the appearance of a protein zone, viz., presumptive phosvitin, immediately ahead of the albumin zone in zone electropherograms of whole sera from estrogenized pullets. The results of the present study tend to support the view that the presumptive phosvitin zone as resolved in veronal–citrate buffer may correspond with Clegg's free electrophoretic 'component 1'.

Certain of the details of the results now reported may have some significance in connection with the interpretation of zone electropherograms of sera from estrogenized or laying fowl in terms of alterations from the levels of components resolved on electropherograms of sera from unestrogenized or immature or male fowl. Although protein-bound phosphorus remained mainly in the lipoprotein zone on zone electropherograms of sera from estrogenized birds in veronal buffer, minor amounts were detected in other zones. Again, even although chelation of calcium led to most of the protein-bound phosphorus moving to the pre-albumin zone, small amounts of protein-bound phosphorus remained in other zones, presumably as contaminants. The peculiar behavior of phosvitin towards staining with Amidoschwarz suggests the desirability of appropriate reservations in the interpretation of densitometric measurements on filter paper electropherograms of sera from laying or estrogenized birds. Phosphoprotein may be present in various zones of paper electropherograms of sera from laying or estrogenized birds without necessarily revealing itself through dyestuff staining procedures, and without obviously changing the resolution of the globulin zones.

Acknowledgments

The authors are indebted to the National Research Council of Canada for a grant-in-aid of research that defrayed the expenses of this work and to Canadian Industries Limited for the C-I-L Fellowship awarded to the senior author.

The authors also wish to thank Professor N. Nikolaiczuk, Macdonald College, for his co-operation in providing suitable experimental birds and facilities; Professor A. L. Carter, Macdonald College, for help with tracer techniques; Messrs. Schering Corporation, Montreal, for generous gifts of hormones; and Dr. W. H. Cook, National Research Council of Canada, for his helpful advice.

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THE PARTICIPATION OF THE LIVER IN THE FORMATION OF POSTHEPARIN ESTERASES[1]

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With the technical assistance of M. PROCHÁZKOVÁ and X. ŠLAISOVÁ

Abstract

The task of the study was to ascertain the participation of the liver in the formation of postheparin esterases. The chronic experiments were carried out on dogs with acrylic vascular cannulas placed into the hepatic vein and vena portae according to London. The modified operation is described in the paper in detail. Heparin was administered in the dose of 200 I.U. per 1 kg body weight into vena portae or one of the hepatic veins. Blood was drawn from these veins at short simultaneous intervals and the activity of postheparin esterases was determined in it. The activity of serum esterases was estimated by the Cherry–Crandall method. Control trials were carried out to study the activity of serum esterases after the administration of saline. The results suggest that postheparin esterases originate in the liver.

It is known that after the intravenous administration of heparin an esterase activity develops in the serum which differs from the clearing factor but which resembles the properties of aliesterase and arylesterase. Details pertaining to this problem, as well as data published in the literature, will be found in a number of papers by Skořepa *et al.* (1, 2, 3, 4, 5). The presence of postheparin esterase in the dog was revealed by Levy and Swank (6). The source and site of formation of this enzyme are unknown so far. In our present work we tried to reveal whether postheparin esterase is formed in the liver.

Method

The investigation was conducted in chronic experiments on dogs. Permanent acryl cannula, similar to those used by London (7), were introduced into the portal vein and one of the hepatic veins. When this method of introducing cannulae is used, it is not necessary to rinse the cannulae and to fill them with heparin, which is indispensable when polyethylene tubes are introduced (8, 9). Accordingly, the procedure is preferable in experiments where heparin is to be used.

The operation was made in one stage. After suturing a part of the right half of the diaphragm to the anterior chest wall, we removed the costal cartilage beginning from the sixth rib. After introducing the cannulae into the veins, we led them to the exterior on the right side of the chest and abdomen, laterally from the operation wound. The cannulae inserted into the hepatic vein were fixed near its orifice into the vena cava caudalis so that damage to the hepatic

[1]Manuscript received April 20, 1961.

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tissue was a minimum. To protect the arterial cannulae against mechanical damage or possible pulling out and to prevent infection from passing along the cannula into the abdominal cavity, we inserted larger cannulae with a firm metal lid between the muscles of the chest wall when inserting cannulae into the hepatic vein, and between the abdominal muscles when inserting cannulae into the portal vein. The inner muscle layer was fixed firmly to the arterial cannula. This technique enabled us to keep the orifice of the cannula and the surrounding tissue completely clean throughout the experiment. Dogs were maintained for 7 or more months with few complications. Those which were observed were rare instances of thrombosis of the arteries, particularly at sites where various substances were injected frequently into the vein and in two instances when the cannula broke in the abdominal cavity.

The withdrawal of blood samples from the cannulated veins without anaesthesia did not produce a response in the dogs.

The serum esterase activity was determined by the method of Cherry and Crandall (10) using ethyl butyrate as substrate. The statistic evaluation was introduced with Student's t test.

Experimental Results

In the first series of experiments on four dogs it was found that administration of heparin (200 units/kg body-weight) into the portal vein produced a marked increase of the esterase activity in the right and left hepatic veins, (on average 170%). The esterase activity in the peripheral blood changed in a similar manner. In further experiments on two dogs we investigated the changes in the esterase activity in the hepatic vein and in the portal vein when blood samples were taken simultaneously from both veins. Although blood samples were withdrawn after longer intervals (35–45 seconds) the values of esterase activity in the hepatic vein were, on an average, 50% higher than in the portal vein. Similar results were obtained in another dog where the interval between the withdrawal of blood samples was substantially reduced. In the first samples, though no substantial changes occurred in the esterase activity in the portal vein, the esterase activity in the hepatic vein increased within 15–20 seconds by as much as 90%, the difference being significant, $P < 0.01$ (Fig. 1). In subsequent samples an increased esterase activity was revealed in the samples from both veins. This finding provided evidence that postheparin esterase is formed probably in the liver; however, it does not rule out the possibility that it is also formed elsewhere, because the higher serum esterase activity in the hepatic vein may be also partly due to esterase formed in other organs. To rule out this possibility, we modified the experiment by injecting heparin into the hepatic vein and subsequently we investigated in three dogs the esterase activity in the portal and hepatic veins simultaneously. From the results of these experiments it is apparent that in the first (and sometimes also the second) blood sample after the administration of heparin the values of esterase activity in the portal vein remain within a normal range,

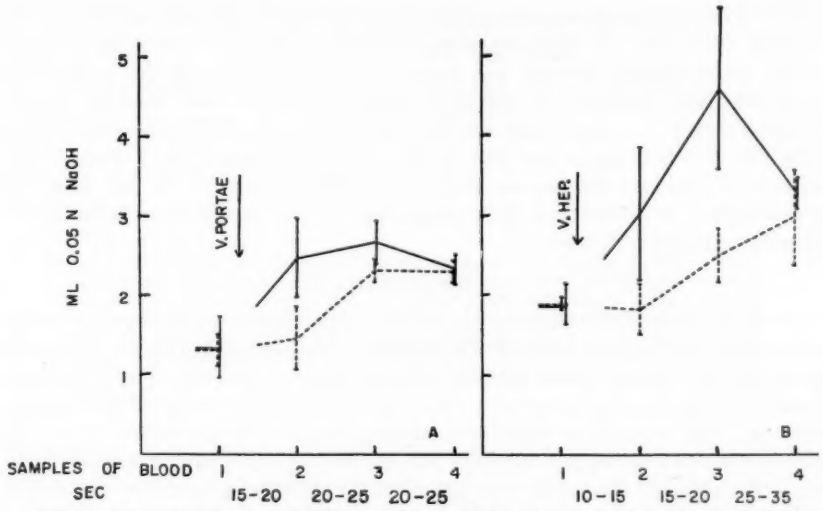

FIG. 1. Change of esterase activity in the portal vein (broken line) and in the hepatic vein (solid line) with standard error of the mean after the administration of heparin: (A) heparin administered into the portal vein; (B) heparin administered into the right hepatic vein.

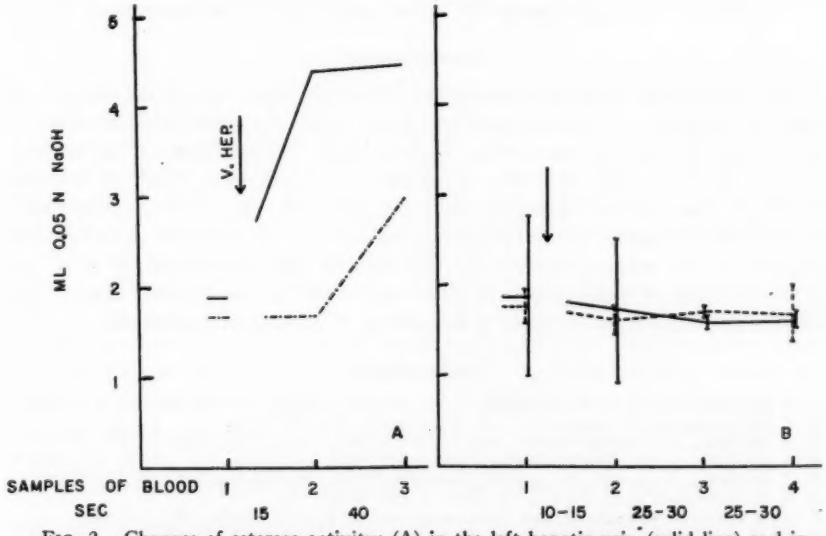

FIG. 2. Changes of esterase activity: (A) in the left hepatic vein (solid line) and in the femoral artery (dot and dash) after the administration of heparin into the left hepatic vein; (B) in the left hepatic vein (solid line) and in the portal vein (broken line) with the standard error of the mean after the injection of physiological saline into the left hepatic vein.

while there is already a more marked increase of the esterase activity in the hepatic vein (Fig. 1). This increase is explained by the flow of heparinized blood which passed through the greater circulation and via the portal vein into the liver. Because no increased esterase values were recorded in the femoral artery at a time when the esterase activity in the hepatic vein is high (Fig. 2), this increase is not due to the inflow of arterial blood with a high esterase content via the hepatic artery. In experiments where we administered physiological saline instead of heparin (Fig. 2) practically no changes in the esterase activity occurred.

Discussion

In the work by Bolková *et al.* (11) the functional difference of liver aliesterase and postheparin serum esterase was studied. The results of this work provide evidence that postheparin esterase differs from aliesterase. This, however, does not rule out the possibility that the liver is the source of postheparin esterase. Our studies, in which we investigated, in chronic experiments, the relationship of postheparin esterase and the liver in dogs with cannulae in the hepatic veins and the portal vein proved that the liver is probably the site of postheparin esterase formation. From the results no conclusions can be drawn so far on the cytological localization of the enzyme in the liver. In view of the speed with which it appears in the circulation it can be assumed that the source of the enzyme is on the inner surface of the capillary bed.

The proof of this hypothesis will be the subject of our subsequent work.

Conclusion

The postheparin esterase content in the hepatic veins and in the portal vein was investigated in chronic experiments on dogs. Heparin was administered into either the portal vein or the hepatic vein. An increase of the esterase activity in the hepatic vein was noted even in experiments where no increase of the esterase activity was yet noted in the portal vein. The administration of physiological saline did not influence the postheparin esterase activity. The increase of the esterase activity in the hepatic vein occurs sooner after the administration of heparin into hepatic vein than in the femoral artery. We assume therefore that the liver is the source of postheparin esterase.

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SYNTHESIS OF ENANTIOMERIC MIXED-ACID GLYCEROLPHOSPHATIDES

I. L-α-(α′-STEAROYL,β-OLEOYL)CEPHALIN AND L-α-(α′-OLEOYL,β-STEAROYL)CEPHALIN[1]

ERICH BAER AND DMYTRO BUCHNEA

Abstract

α′-Stearoyl,β-oleoyl L-α-glycerylphosphorylethanolamine and α′-oleoyl,β-stearoyl L-α-glycerylphosphorylethanolamine were synthesized by phosphorylating D-α′-stearoyl,β-oleoylglycerol and D-α′-oleoyl,β-stearoylglycerol with phosphorus oxychloride and quinoline, esterifying the resulting mixed-acid phosphatidic acid dichlorides with 2′-hydroxyethylphthalimide in the presence of pyridine, and removing the protective phthaloyl group of the α′-stearoyl,β-oleoyl-, and α′-oleoyl,β-stearoyl-L-α-glycerylphosphoryl-2′-hydroxyethylphthalimides by hydrazinolysis.

Glycerol phosphatides containing two identical fatty acid substituents, whether saturated or unsaturated, have been isolated from natural sources in a few instances only. In general, natural phosphatides appear to possess two dissimilar fatty acid substituents, one of which, in most cases, is unsaturated. Thus the purely chemical synthesis of phosphatides possessing the α structure and L configuration of natural glycerolphosphatides (1–8), and having two dissimilar fatty acid substituents, one being unsaturated, has been one of the outstanding major problems remaining in this field.

Recently, de Haas and van Deenen (9), and van Deenen, de Haas, Heemskerk, and Meduski (10), have reported lipolytic studies of synthetic γ-stearoyl-β-oleoyl L-α-phosphatidyl ethanolamine ($[\alpha]_D$ +6.0°) and γ-oleoyl-β-stearoyl L-α-phosphatidyl ethanolamine ($[\alpha]_D$ +6.1°). No experimental details for the preparation of the two mixed-acid cephalins were given, other than that they were made according to a modification of the method applied to the synthesis of mixed-acid lecithins (11). In the present communication we describe the synthesis of the same cephalins by a different method.

The synthesis of a mixed-acid phosphatide containing a saturated and unsaturated fatty acid, according to our method, requires as starting material one enantiomeric form of a mixed-acid α,β-diglyceride. A procedure which permits the synthesis of the four theoretically possible isomeric forms of a mixed-acid α,β-diglyceride was developed in this laboratory by Buchnea (12). Each is obtainable as a pure compound devoid of any contamination by positional or spatial isomers. The fatty acid substituents may consist either of two dissimilar saturated fatty acids or a saturated fatty acid and oleic acid.

[1]Manuscript received July 12, 1961.
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The procedure was applied to the synthesis of D-α'-stearoyl,β-oleoylglycerol (I) and D-α'-oleoyl,β-stearoylglycerol (I*a*), the first pair of positional isomers of mixed-acid diglycerides to be obtained in enantiomeric form (12).

Having made accessible the spatial as well as positional isomers of mixed-acid α,β-diglycerides, we were now able to undertake the synthesis of both positional isomers of mixed-acid L-α-glycerolphosphatides (IV and IV*a*) containing as substituents a saturated and an unsaturated fatty acid. The reaction scheme (Fig. 1) illustrates our procedure for the synthesis of L-α-(α'-stearoyl,β-oleoyl)cephalin and L-α-(α'-oleoyl,β-stearoyl)cephalin. It is the

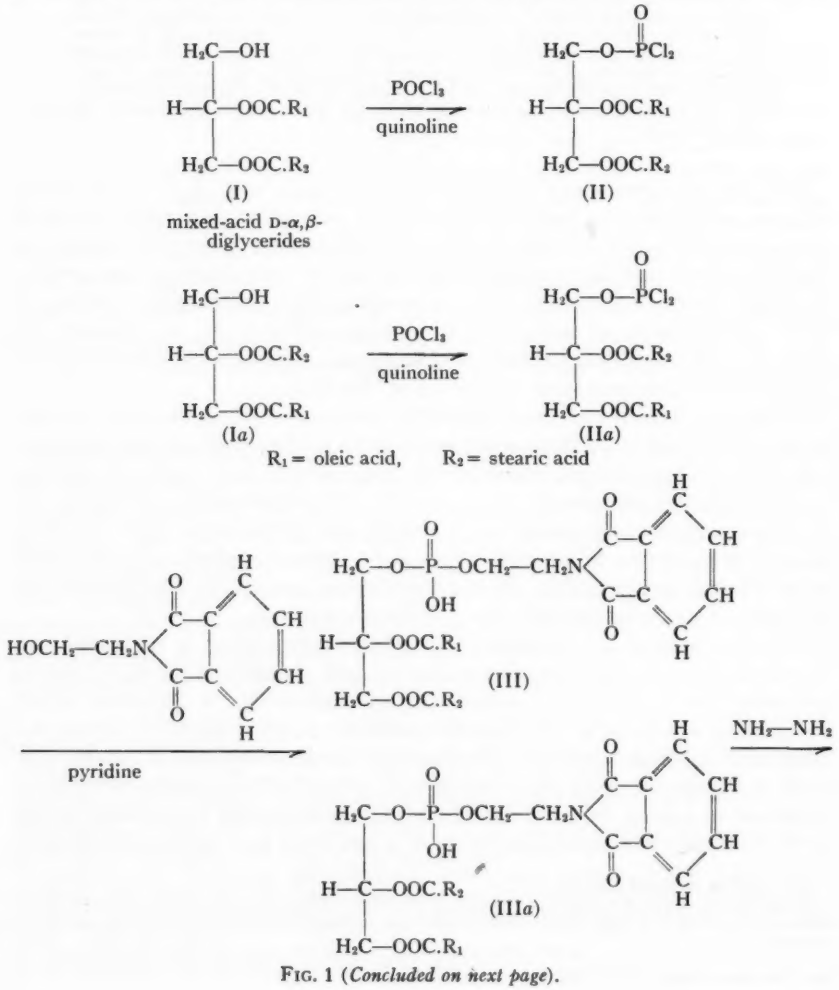

FIG. 1 (*Concluded on next page*).

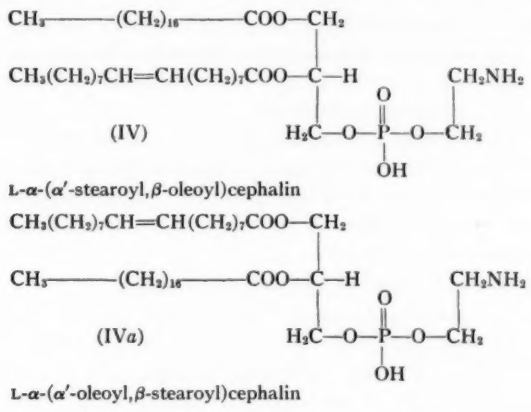

FIG. 1. Synthesis of mixed-acid cephalins.

same procedure by means of which we obtained L-α-(dioleoyl)cephalin (7), except that mixed-acid D-α,β-diglycerides are substituted for D-α,β-diolein.

To obtain the mixed acid cephalins (IV and IV*a*), α′-stearoyl,β-oleoylglycerol (I) and α′-oleoyl,β-stearoylglycerol (I*a*) were phosphorylated with phosphorus oxychloride and quinoline. The resulting mixed-acid phosphatidic acid dichlorides (II and II*a*) were esterified with N-phthaloylethanolamine in the presence of pyridine, and the N-phthaloylcephalins (III and III*a*) were freed of their protective phthaloyl groups by hydrazinolysis.

The simultaneous presence of bound oleic acid and stearic acid, among other acids, in mixtures of natural cephalins makes it a strong possibility that either L-α-(α′-stearoyl,β-oleoyl)cephalin or L-α-(α′-oleoyl,β-stearoyl)cephalin, possibly both, will be found to be naturally occurring glycerol phosphatides.

Since the starting materials, i.e. mixed-acid α,β-diglycerides, were pure compounds, both structurally and optically, and since their phosphorylation proceeds without racemization, the mixed-acid cephalins are obtained as structurally and optically pure compounds. Because they contain stearic acid and oleic acid, two of the most widely occurring fatty acids in nature, they are ideal substrates for studies of the specificity of phospholipases. Such investigations are being carried out at the present time in collaboration with Dr. Morris Kates of the National Research Council of Canada.

Experimental

Materials

D-α′-Stearoyl,β-oleoylglycerol and D-α′-oleoyl,β-stearoylglycerol were prepared as described by Buchnea and Baer (12), using oleic acid (13) and stearic acid with a purity of at least 99.9%. The 2′-hydroxyethylphthalimide was obtained by the procedure of Rose (14), and was recrystallized from water;

m.p. 126.5°–127.5°. Chloroform was dried and freed of ethanol immediately before use by distillation over phosphorus pentoxide. Anhydrous pyridine was prepared from the best commercial grade of pyridine, by refluxing it over barium oxide, and distilling it with the exclusion of moisture. Anhydrous quinoline was prepared from synthetic quinoline by shaking it for 2 days with barium oxide, and distilling the quinoline *in vacuo* under anhydrous conditions. The cyclohexane was dried with metallic sodium. The silicic acid was Mallinckrodt (Analytic Reagent, 100 mesh). The phosphorus oxychloride was purified by fractional distillation.

α′-Stearoyl,β-oleoyl L-*α-Glycerylphosphorylethanolamine* (*IV*)

Phosphorylation.—In an oven-dried 200-ml three-necked round flask with ground joints, and equipped with an oil-sealed mechanically driven stirrer, calcium chloride tube, and dropping funnel, was placed 1.55 g (10 mmoles) of phosphorus oxychloride. The flask was immersed in an ice bath, and to the vigorously stirred phosphorus oxychloride was added dropwise a solution of 6.25 g (10 mmoles) of D-α′-stearoyl,β-oleoylglycerol (I) and 1.5 g (11.6 mmoles) of quinoline in 15 ml of cyclohexane over a period of 90 minutes. The temperature of the reaction mixture then was raised to 25° C and the stirring was continued. After 1 hour, the temperature of the mixture was lowered to 10° C, and a solution of 1.9 g (10 mmoles) of 2′-hydroxyethylphthalimide and 3 ml of pyridine in 35 ml of chloroform was added over a period of 30 minutes. After the mixture had been kept for 2 hours at 25° C, two drops of distilled water were added, and the mixture was allowed to stand for 90 minutes at this temperature. At the end of this period it was diluted with 200 ml of ether, filtered with suction, and the filter residue was rinsed with three 15-ml portions of ether. The combined filtrates were washed as rapidly as possible with three 100-ml portions of ice-cold 2 *N* sulphuric acid, two 100-ml portions of a saturated sodium bicarbonate solution, and two 100-ml portions of water. To break the rather stable emulsions which form on washing with water, 3 ml of ethanol was added, and the mixtures were centrifuged. The ethereal solution was dried with 50 g of anhydrous sodium sulphate, filtered, and concentrated under reduced pressure to a viscous oil. The residue was redissolved in 250 ml of petroleum ether (b.p. 35°–60°), the solution was kept at −10° C for 15 hours, and cleared by centrifugation in a refrigerated centrifuge at −10° C. The solvent was distilled off under reduced pressure, and the residue was dried to constant weight in a vacuum of 0.02 mm. The remaining L-α-(α′-stearoyl,β-oleoyl)-N-phthaloylcephalin (III), a viscous oil, weighed 7.0 g (79.7% of theory) and was pure enough for the preparation of L-α-(α′-stearoyl,β-oleoyl) cephalin.

Removal of the Protective Phthaloyl Group

To a vigorously stirred solution of 7 g of L-α-(α′-stearoyl,β-oleoyl)-N-phthaloylcephalin in 130 ml of ethylene glycol monomethyl ether, which was kept at 8° C (±1°), was added over a period of 1 hour a 0.5 *N* aqueous solution

of sodium hydroxide until a pH of 7 was reached. Approximately 17 ml was required. This was followed by the addition of a solution of 0.29 g of hydrazine (100%) in 0.5 ml of water over a period of 10 minutes. The temperature of the continuously stirred reaction mixture then was gradually raised from 8° to 60° C within 2 hours, and kept at this temperature for another hour. The solvents then were distilled off in the vacuum of a water pump, and the residue was thoroughly dried at 0.1–0.01 mm and a bath temperature of 40° C. The material was extracted with three 50-ml portions of warm ether, the combined ether extracts were cleared by centrifugation, and concentrated under reduced pressure to about one-third of the original volume. To the concentrate were added 1.5 ml of water, 4 ml of methanol, and 25 g of Amberlite IRC-50 (H^+ form), and the mixture was shaken for 30 minutes. The Amberlite was filtered off, washed thoroughly with ether, and the combined filtrates were evaporated under reduced pressure. The residue was dried to constant weight in a vacuum of 0.1 mm. The crude cephalin, weighing 5.6 g, was dissolved in 100 ml of chloroform, and the solution was passed through a column of 300 g of silicic acid (approximately 3.5 cm in width and 60 cm in height) which had been prepared as described elsewhere by the present authors (7). The column then was washed with chloroform (U.S.P.) until the effluent was free of solute. Approximately 500 ml of solvent was required. This was followed by a mixture of chloroform and methanol (4:1, v/v). The first 300 ml of eluate (A) contained 1.5 g of material low in nitrogen and phosphorus; the next 200 ml of eluate (B) contained 1.5 g of fairly pure cephalin. The final 200 ml of eluate (C) contained pure cephalin. The eluates were evaporated under reduced pressure, and the residues were kept in a vacuum of 0.1–0.01 mm at a bath temperature of 35°–40° C until their weights were constant. The L-α-(α′-stearoyl,β-oleoyl)-cephalin obtained from eluate C weighed 1.6 g (27% of theory based on phthaloylcephalin). The yield of pure cephalin was increased considerably by rechromatographing the cephalin obtained from eluate B. The L-α-(α′-stearoyl,β-oleoyl)cephalin is a white, solid material, which at room temperature is highly soluble in chloroform, or ether, and sparingly soluble in anhydrous acetone, $[\alpha]_D$ +6.0° in ethanol-free chloroform (c, 10). Anal. Calc. for $C_{41}H_{80}O_8NP$(746): C, 66.01; H, 10.81; N, 1.88; P, 4.15; iodine no. 34.0. Found: C, 66.06; H, 11.08; N, 1.90; P, 4.20; iodine no. 35.5. R_f 0.76 (on silicic acid impregnated Whatman No. 1 paper and a solvent mixture of 2,6-dimethyl-4-heptanone, glacial acetic acid, and water, 40:25:5, v/v).

L-α-(α′-Oleoyl,β-stearoyl)cephalin (IVa)

Its synthesis was carried out as described above for its isomer. The phosphorylation of 6.25 g of D-α′-oleoyl,β-stearoylglycerol (I*a*), and esterification of the resulting mixed-acid phosphatidic acid dichloride (II*a*) with 1.9 g of 2′-hydroxyethylphthalimide, gave 7.2 g (82.6% of theory) of N-phthaloyl-cephalin (III*a*). Its hydrazinolysis, and separation of the reaction products by column chromatography on silicic acid, gave about 1 g of fairly pure cephalin

(eluate B), and 2.0 g (32.7% of theory) of pure L-α-(α′-oleoyl,β-stearoyl)-cephalin (eluate C). The yield of pure cephalin again could be raised considerably by rechromatography of the cephalin obtained from eluate B, $[\alpha]_D$ +6.0° in ethanol-free chloroform (*c*, 10). R_f 0.76. Anal. Calc. for $C_{41}H_{80}O_8NP$ (746): C, 66.01; H, 10.81; N, 1.88; P, 4.15; iodine no. 34.0. Found: C, 66.53; H, 11.17; N, 1.75; P, 4.15; iodine no. 34.8.

Both cephalins were chromatographically homogeneous substances. They possessed very similar solubility properties, and their infrared spectra recorded in chloroform solution (*c*, 10) on a Beckman IR-5 infrared spectrophotometer (path of cell 0.093 mm) were undistinguishable from each other, and from L-α-(dioleoyl)cephalin. Their melting points were determined in sealed-off capillary tubes filled with nitrogen, using an electrically heated bath of *n*-butyl phthalate and short-stem thermometers. The L-α-(α′-stearoyl,β-oleoyl)-cephalin began to sinter at 115° C, turned amber at 155° C, and coalesced with the formation of a meniscus at 182°–183° C. The L-α-(α′-oleoyl,β-stearoyl)-cephalin sintered slightly at 80° C, turned amber at 160° C, and coalesced suddenly with the formation of a meniscus at 181°–182° C.

Acknowledgment

The work was assisted with funds granted by the Medical Research Council of Canada.

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NOTES

THE INFLUENCE OF DIETARY VITAMIN B_{12} ON THE HEPATIC VITAMIN B_{12} BIOLOGIC HALF-LIFE OF SWINE*

G. L. FREDERICK

A commercial sow diet that contained 1 to 2 μg of vitamin B_{12}/lb was fed to five sows for approximately 8 months. The vitamin B_{12} was derived chiefly from fish meal and meat meal which were both present in the diet at levels of 1%. The same diet, fortified with 49 μg of crystalline vitamin B_{12}/lb, was fed to another group of eight sows for the same period of time. At the close of this 8-month period all the sows were given one feeding of a vitamin-B_{12}-deficient diet (1), and 0.5 to 1 hour later they were given 200 μg of Co^{60} – vitamin B_{12} (approximately 0.9 μc) via stomach tube. After this treatment the sows in the first group were fed their original diet for 21 days. Then the vitamin-B_{12}-deficient feedings and radioactive doses were repeated. Sows in the second group were fed the vitamin-B_{12}-supplemented diet for 40 days prior to their second radioactive doses. The respective diets were fed for time

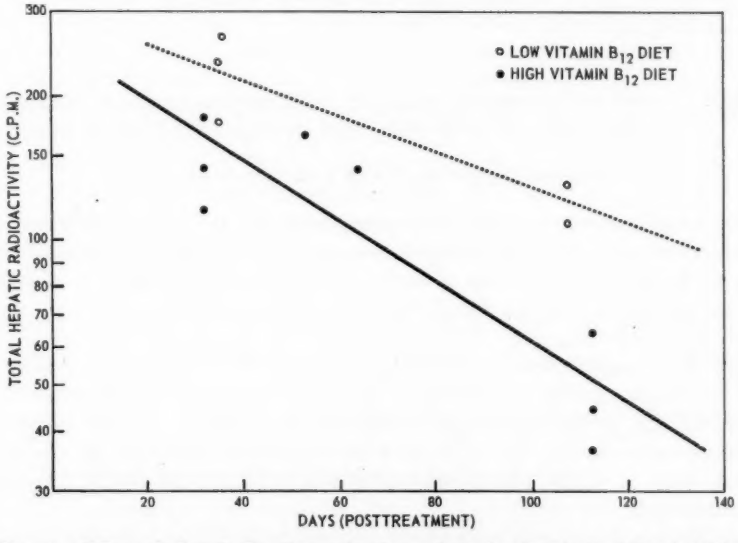

FIG. 1. Effect of vitamin B_{12} dietary intake on hepatic vitamin B_{12} biologic half-life.

*Contribution No. 71 from the Animal Research Institute, Canada Department of Agriculture.

periods of variable duration after the second doses, after which the sows were slaughtered and their livers were removed. Total hepatic radioactivity was determined in vitro with a scintillation counter and gamma-ray spectrometer. It was assumed that the decline in hepatic radioactivity within a dietary group was indicative of the group's hepatic vitamin B_{12} biologic half-life. (Glass and Mersheimer (2) studied the hepatic biologic half-life of individual dogs by means of successive surface scintillation counts and also liver biopsies.)

The influence of time and diet following the second dose is shown in Fig. 1. The slopes of the weighted regressions were significantly different ($P < 0.05$). The biologic half-life of vitamin B_{12}, as indicated by this method, was longer in the livers of the sows fed the low vitamin B_{12} diet (approximately 81 days compared with 48 days). Because the total vitamin B_{12} content in the livers of the sows fed the low vitamin B_{12} diet would be less than, or only equal to, that in the livers of the sows fed the high vitamin B_{12} diet, therefore the hepatic vitamin B_{12} turnover was smaller in the animals that received the low vitamin B_{12} diet.

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RECEIVED MAY 1, 1961.
ANIMAL RESEARCH INSTITUTE,
RESEARCH BRANCH,
CANADA DEPARTMENT OF AGRICULTURE,
OTTAWA, ONTARIO.

THE FAILURE TO PRODUCE SOMATIC AND IMMUNOLOGICAL CHANGES IN INBRED AKR MICE BY SPECIFIC DEOXYRIBONUCLEIC ACID

VIKTOR HOLOUBEK* AND LUBOMIR HNILICA†

The experimental transfer of genetic character by deoxyribonucleic acid (DNA) represents a new path in biochemical genetics. Whilst many microbial systems have been described in which successful transformation of specific biochemical properties by DNA has been shown, there is only one similar experiment of Benoit *et al.* (1, 2) carried out in vertebrates. All attempts to repeat these experiments in duckling (3) or to produce similar changes on unhomogeneous genetical material in sea urchin (4) or rat (5, 6) have failed. However, this problem is of such biological importance that we decided to repeat Benoit's experiment in inbred mice because of their high genetic homogeneity and to follow the possible genetical changes in three subsequent generations.

The DNA was prepared from spleen of three inbred strains of mice according to Schwander and Signer (7). The DNA donor strains were DBA mice (DNA

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N/P = 1.79, E_{max} at 259 mμ, ϵ_P = 7030), Balb mice (DNA N/P = 1.77, E_{max} at 258 mμ, ϵ_P = 6950), and C_3H mice (DNA N/P = 1.77, E_{max} at 260 mμ, ϵ_P = 7000). In each of the three groups 30 newborn animals of AKR strain of mice were injected intraperitoneally with DNA solution in physiological saline starting with 0.04 ml per animal on the first day and rising daily up to 0.4 ml on the 15th day after birth. The total amount of injected DNA was 48 mg per mouse.

To demonstrate that the DNA used in our experiments was probably not degraded during the isolation procedure, an identical preparation was made from spleens mixed with a culture of *Diplococcus pneumoniae* strain Ph 3. The transforming activity of the isolated DNA mixture was tested in the R form of *D. pneumoniae* strain PMS II (8). The transforming activity of the *D. pneumoniae* strain Ph 3 DNA was not affected during the isolation procedure.

All mice injected with DNA were inbred and the further inbred propagated offspring were followed for three subsequent generations. The pigmentation and shape of the body were observed; the possible change in histocompatibility was followed using transplantation of skin grafts (9). No changes occurred in the pigmentation or in shape of the body of the AKR albino strain injected with DNA or in their progeny. The skin grafts transplanted from DNA donor strains to all AKR mice used in this experiment were rejected.

The failures to produce transformation by injecting higher animals with specific DNA may be due to enzymatic destruction of injected DNA by the deoxyribonuclease present in the animals (6) or to the low incidence of transformation. Even in such primitive organisms as bacteria this incidence is very low. But the success of Fahmy in producing mutations in *Drosophila* by injecting the adults with non-specific DNA (private communication) and the production of tumors both in mice (10) and fish (11) with non-specific herring sperm DNA leave the question open whether in Benoit's experiment the genetic effect could not be an example of unspecific mutation produced by DNA rather than a transfer of specific genetic characters by parts of donator's DNA coding system.

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Received April 17, 1961.
The Cancer Research Institute,
Bratislava,
Czechoslovakia.

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CONTENTS

Page

Corrections i

BIOCHEMISTRY

D. J. Ecobichon and W. Kalow—Some properties of the soluble esterases of human liver 1329

D. W. A. Roberts—Hydrolysis of some deoxyribonucleosides by wheat leaf juice 1333

U. H. M. Fagerlund and D. R. Idler—Marine sterols. IX. Biosynthesis of 24-methylenecholesterol in clams 1347

J. R. Bennett and N. H. Tattrie—The acylation of monoacyl-L-α-glycerylphosphorylcholine under various conditions 1357

V. N. Nigam, H.-G. Sie, and William H. Fishman—Distribution in nature of heptulose-phosphate-forming systems 1367

K. K. Govind Menon, C. C. Lucas, and W. G. B. Casselman—The estimation of fibrous connective tissue in liver by a chemical method 1373

D. C. Jessup and G. S. Wiberg—Effects of incubation conditions on the in vitro assay method for insulin 1381

B. A. Bohm, R. K. Ibrahim, and G. H. N. Towers—The partial elucidation of the structure of a new coumarin from *Hydrangea macrophylla* Ser. 1389

J. E. Troelsen—Determination of soluble carbohydrates from the absorbances of sulphonated 1-naphthol derivatives of hexoses and pentoses. 1397

W. G. Martin—Molecular weight of bovine plasma albumin and vitellenin in formic acid 1405

Edmond R. Cole and Edwin T. Mertz—Studies on plasminogen. I. Further purification of bovine plasminogen 1419

Norman Kalant, Reuben Schucher, and William C. Galley—Effect of antibodies on removal of insulin from the circulation 1433

R. H. Common, A. J. Moo-Young, and K. A. McCully—Some effects of dietary thiouracil and dietary iodine supplementation on the responses of the immature pullet to estrogen 1441

K. A. McCully and R. H. Common—Zone electrophoresis of protein-bound phosphorus of fowl's serum 1451

Erich Baer and Dmytro Buchnea—Synthesis of enantiomeric mixed-acid glycerolphosphatides. I. L-α-(α′-Stearoyl,β-oleoyl)cephalin and L-α-(α′-oleoyl,β-stearoyl)cephalin 1471

Notes:

G. L. Frederick—The influence of dietary vitamin B_{12} on the hepatic vitamin B_{12} biologic half-life of swine 1477

Viktor Holoubek and Lubomir Hnilica—The failure to produce somatic and immunological changes in inbred AKR mice by specific deoxyribonucleic acid 1478

PHYSIOLOGY

J. Kohout, L. Korbová, and J. Skořepá. With the technical assistance of M. Procházková and X. Šlaisová—The participation of the liver in the formation of postheparin esterases 1467

PHARMACOLOGY

Surendra S. Parmar, Morley C. Sutter, and Mark Nickerson—Localization and characterization of cholinesterase in subcellular fractions of rat brain and beef pituitary 1335